

## **Mesophasic organization of GABA<sub>A</sub> receptors in hippocampal inhibitory synapse**

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1     **Abstract:**

2     Information processing in the brain depends on synaptic transmission and plasticity, which in  
3     turn require specialized organization of neurotransmitter receptors and scaffolding proteins  
4     within the postsynaptic density (PSD). However, how these molecules are organized *in situ*  
5     remains largely elusive, limiting our mechanistic understanding of synaptic formation and  
6     functions. Here, we have developed template-free classification of over-sampled sub-  
7     tomograms to analyze cryo-electron tomograms of hippocampal synapses, enabling us to  
8     identify type-A  $\gamma$ -aminobutyric acid receptor (GABA<sub>A</sub>R) in inhibitory synapses and determine  
9     its *in situ* structure at 19 Å resolution. We found that these receptors are organized  
10    hierarchically: from GABA<sub>A</sub>R super-complexes with a fixed 11-nm inter-receptor distance but  
11    variable relative angles, through semi-ordered two-dimensional receptor networks with  
12    reduced Voronoi entropy, to mesophasic assembly with a sharp phase boundary. This  
13    assembly aligns with condensates of postsynaptic scaffolding proteins and putative  
14    presynaptic vesicle release sites. Such mesophasic self-organization may allow synapses to  
15    achieve a “Goldilocks” state with a delicate balance between stability and flexibility, enabling  
16    both reliability and plasticity in information processing.

17 **Introduction**

18 Neuronal synapses are intricate communication devices, operating as fundamental building  
19 blocks underlying virtually all brain functions<sup>1-4</sup>. An essential part of the synapse is the lipid-  
20 bound, proteinaceous postsynaptic density (PSD), in which neurotransmitter receptors and  
21 other synaptic proteins are concentrated<sup>5-8</sup>. The specialized organization of the PSD is  
22 critical for the efficacy of synaptic transmission<sup>9,10</sup>. Meanwhile, the reorganization of  
23 receptors and other PSD proteins is widely known as a mechanism of synaptic plasticity,  
24 which in turn underlies many cognitive functions such as learning and memory<sup>11,12</sup>.

25 Different forms of PSD organization have been proposed, including meshwork based on  
26 electron microscopy and biochemical assays<sup>13-15</sup>, nano-domains based on super-resolution  
27 optical imaging<sup>9,10,16-18</sup>, and liquid condensate based on *in vitro* PSD mixing assay<sup>19,20</sup>.  
28 However, the PSD is heterogeneous and pleomorphic, and their protein components are  
29 small in size, presenting considerable challenges for resolving its molecular organization.  
30 For example, even super-resolution optical imaging can only describe synaptic organizations  
31 at the precision of protein clusters with its ~20 nm resolution<sup>16,17,21</sup>. Electron microscopy,  
32 although with higher resolution, lacks molecular specificity, thus hindering the ability to  
33 identify synaptic receptors and other proteins inside synapses. These synaptic molecules,  
34 such as type-A  $\gamma$ -aminobutyric acid receptors (GABA<sub>A</sub>Rs), are often small and surrounded  
35 by the crowded cellular environment. Consequently, how individual PSD molecules are  
36 organized *in situ* is largely unknown, limiting our understanding of molecular mechanisms  
37 underlying synaptic formation and functions.

38 Here, we employed the state-of-the-art cryo electron tomography (cryoET) with Volta  
39 phase plate and direct electron detector to obtain structures of neuronal synapses in their  
40 native conditions. In order to automatically identify neurotransmitter receptors inside  
41 synapses without the need of labeling, we developed a method of template-free  
42 classification with uniformly oversampled sub-tomograms on the membrane. With this  
43 method, we obtained an *in situ* structure of GABA<sub>A</sub>R at 19 Å resolution and discovered a

44 hierarchical organization of GABA<sub>A</sub>Rs within the PSD, establishing the structural basis for  
45 synaptic transmission and plasticity.

46

## 47 **Results**

48 **Identification of GABA<sub>A</sub>Rs by oversampling and template-free classification.** To  
49 understand the molecular organization of GABA<sub>A</sub>Rs *in situ*, we imaged synapses of cultured  
50 hippocampal neurons using cryoET with Volta phase plate (Supplementary Video 1). Taking  
51 advantage of correlative microscopy, we have shown that a thin sheet-like density parallel to  
52 the postsynaptic membrane is a defining feature of GABAergic inhibitory synapses<sup>7</sup> (Fig.  
53 1a). Following this criterion, we identified that 72 synapses in the tomograms we obtained  
54 are inhibitory synapses. Many particles visualized on the postsynaptic membrane in these  
55 synapses have shapes characteristic of pentameric GABA<sub>A</sub>R<sup>22</sup> (Figs. 1b-e; Supplementary  
56 Video 2), which is the most abundant membrane protein species in GABAergic synapses<sup>23,24</sup>  
57 (Extended Data Table 1). We thus assigned these particles as GABA<sub>A</sub>Rs on the native  
58 postsynaptic membrane. To automate the unbiased identification of GABA<sub>A</sub>Rs, we devised a  
59 systematic approach that uses oversampling of sub-tomograms to ensure inclusion of all  
60 particles existing on the postsynaptic membranes, and then classifies the oversampled sub-  
61 tomograms with template-free, Bayesian 3D classification method as implemented in  
62 Relion<sup>25</sup> to sort out GABA<sub>A</sub>R particles from all the particles (Extended Data Figs. 1-4; see  
63 also Methods). The structure of GABA<sub>A</sub>R emerged during the iterative classification  
64 (Extended Data Fig. 1c). After eliminating duplicates, we sorted out 9,618 GABA<sub>A</sub>Rs from all  
65 72 synapses (Extended Data Fig. 1b) and placed them back on the postsynaptic membranes  
66 to visualize their spatial distribution (Fig. 1d). After 3D refinement, a sub-tomogram average  
67 of *in situ* GABA<sub>A</sub>R was obtained at 19 Å resolution (Fig. 1f-g).

68

69 ***In situ* structure of GABA<sub>A</sub>R.** The sub-tomogram average of GABA<sub>A</sub>R was ~11 nm in  
70 length and ~7 nm in width and had a central pore (Fig. 1f). Overall, our *in situ* structure  
71 matched the previously characterized structure of reconstituted GABA<sub>A</sub>R<sup>22</sup>, except that extra

72 densities were found at the edges of the extracellular domain (Fig. 1f). These extra densities  
73 might represent additional glycans only existing in the native proteins expressed in  
74 neurons<sup>26</sup>. Densities for the membrane bilayer were also well resolved (Fig. 1f). The rough  
75 shape of the density for the transmembrane helices matched the atomic models of the  
76 reconstituted GABA<sub>A</sub>Rs<sup>22,26-29</sup>, with some slight difference that could be due to averaging of  
77 different subunits (Fig. 1f; Extended Data Fig. 5a). The intracellular loops (~500 a.a. for 5  
78 subunits, missing in atomic structures) were not observed in our reconstruction even at low  
79 threshold (Extended Data Fig. 5b), suggesting that those loops are intrinsically flexible even  
80 though they are likely to bind to postsynaptic scaffolding proteins *in situ*.

81

82 **Super-complex of GABA<sub>A</sub>Rs.** With the GABA<sub>A</sub>Rs identified *in situ*, we next investigated  
83 their spatial organization on the postsynaptic membrane. By measuring the distance of each  
84 receptor to its neighbors, we found that the distributions of the first and the second nearest  
85 neighbor distances both peaked sharply at ~11 nm (Fig. 2a-b), indicating that GABA<sub>A</sub>Rs tend  
86 to maintain a fixed distance with their neighboring receptors. Receptor concentration  
87 measured as number of particles/μm<sup>2</sup> within the concentric rings around GABA<sub>A</sub>Rs also  
88 peaked at ~11 nm (Fig. 2c), further supporting that 11 nm is a characteristic inter-receptor  
89 distance (IRD). At this distance, the concentration of GABA<sub>A</sub>R reached ~4,000 μm<sup>-2</sup>, which  
90 was about twice of the plateau level that occurs just 5 nm away (Fig. 2c). This characteristic  
91 11-nm IRD was consistently found in most (64 out of 72) synapses (Fig. 2d). The rest had  
92 generally fewer receptors and larger median IRDs (Fig. 2d), probably due to their immaturity  
93 in early synapse development. By selecting receptors and their neighboring receptors with  
94 11±4 nm IRDs (Fig. 2e). we obtained a sub-tomogram average of GABA<sub>A</sub>R super-complex  
95 consisting of a pair of receptors (Fig. 2f). Moreover, classification of oversampled sub-  
96 tomograms without symmetry also yielded a class with a pair of receptor-like particles with  
97 ~11 nm IRD (Extended Data Fig. 4). Thus, this IRD imposes a stringent constraint on the  
98 organization of GABA<sub>A</sub>Rs on the inhibitory postsynaptic membrane. In the averaged receptor  
99 pair super-complex, the pseudo 5-fold symmetry in both receptors was lost, suggesting that

100 the relative rotation of each receptor was less constrained (Fig. 2f). Indeed, the distribution  
101 of the in-plane rotation angle (denoted as angle  $\omega$ ) of a receptor relative to the receptor pair  
102 axis was quite uniform (Fig. 2g). Reconstructing the receptor pairs with specific  $\omega$  angles  
103 clearly restored the pseudo-5-fold symmetry of the corresponding receptor (Fig. 2h).

104 One GABA<sub>A</sub>R could also pair with two other receptors, forming a receptor triplet (Figs.  
105 2e, i). In the triplet structure, whereas the distances between the neighboring receptors were  
106 constrained to ~11 nm, the angle (denoted as angle  $\theta$ ) between the two arms of the triplet  
107 was unrestricted, with a rather uniform distribution ranging from 60° to 180° (Fig. 2i). The  
108 structures of receptor triplets with different  $\theta$  angles could also be reconstructed (Fig. 2j).  
109 Thus, the near-neighbor organization of GABA<sub>A</sub>Rs is morphologically flexible with variable  $\omega$   
110 or  $\theta$  angles, but topologically invariable with fixed IRD. This unique feature is characteristic  
111 of a mesophasic state, which is neither liquid that does not maintain inter-molecule distance,  
112 nor crystalline that has constant crystal angles.

113  
114 **Two-dimensional networks of GABA<sub>A</sub>Rs.** In addition to pairs and triplets of “linked”  
115 receptors, many receptors (26.1%) in fact had more than two 11-nm neighbors (Fig. 3a), and  
116 further organized into two-dimensional networks of various sizes and shapes (Fig. 3b). In the  
117 meantime, 20.0% receptors did not integrate into the network, hereafter defined as solitary  
118 receptors (Figs. 3a-b). The proportion of solitary receptors and mean size of the networks  
119 were independent of postsynaptic area or number of receptors in a synapse (Fig. 3c;  
120 Extended Data Fig. 6a), consistent with the idea that the function of these synapses could be  
121 altered independently either by changing number of receptors or by modifying the  
122 organization of postsynaptic protein network<sup>30</sup>.

123 Intriguingly, the mean size of receptor networks in a synapse, when plotted against  
124 receptor concentration, was always larger than that for simulated randomly distributed  
125 receptors (RDR) or randomly distributed receptors without overlap (RDR\*) (Fig. 3d).  
126 Furthermore, the overall distribution of network size followed power law (Fig. 3e; Extended  
127 Data Fig. 6b). The power-law exponent (1.87), representing the fractal dimension of

128 receptor networks, was smaller than that for RDR (2.44) and RDR\* (2.40) (Fig. 3e). These  
129 results suggest that receptor networks tend to “attract” more receptors to grow into larger  
130 networks, a property typically found in self-organizing processes near critical states<sup>31</sup>.

131 To quantify the degree of orderliness for the receptor organization, we calculated  
132 Voronoi entropy that measures information content in the Voronoi tessellation of the receptor  
133 localizations<sup>32</sup> (Extended Data Fig. 6c). The Voronoi entropy becomes zero for a perfectly  
134 ordered structure, while for a fully random 2D distribution of points the value has been  
135 reported to be 1.71<sup>33</sup>. The Voronoi entropy for our measured receptor distribution was 1.50,  
136 smaller than that for RDR (1.60) and RDR\* (1.55) (Fig. 3f). The smaller entropy for the  
137 measured receptors is likely to arise from the semi-ordered 2D networks. This Voronoi  
138 entropy value in between the entropy of crystal and liquid further suggests that the receptors  
139 organize in mesophasic state. This mesophasic state is apparently much more disorder than  
140 the liquid-crystalline state of acetylcholine receptors in neuromuscular junction<sup>34,35</sup>. This  
141 could potentially allow for rapid change in receptor organization to serve as a plasticity  
142 mechanism in GABAergic synapses. Several synapses (12.9%) had Voronoi entropy larger  
143 than that of RDR (Fig. 3g). They are mostly synapses with fewer receptors that were unable  
144 to establish semi-ordered organization.

145

146 **Mesophasic assembly of inhibitory PSD.** The semi-ordered receptor networks  
147 presumably reflect a mesophasic state of the self-organized PSD. If this is the case, one  
148 would expect that the mesophasic PSD may separate from its aqueous environment with a  
149 phase boundary. To test this, a smoothed convex hull of all linked receptors (Fig. 4a1;  
150 Extended Data Figs. 7a-b) was constructed. Within this hull that enclosed about 66% of the  
151 postsynaptic membrane area (Extended Data Fig. 7c), the receptor concentration was high  
152 (~3,000  $\mu\text{m}^{-2}$ ) and relatively uniform. This concentration dropped steeply within ~18 nm  
153 across the hull (Fig. 4b). Thus, the smoothed convex hull can indeed be considered as the  
154 phase-separating boundary of the mesophasic receptor assembly. Interestingly, the sharp  
155 boundary was characteristic only for the linked receptor, whereas the concentration of the

156 solitary receptors changed only moderately across the convex hull (Fig. 4b). Thus, the  
157 solitary receptors appear to diffuse more readily into and out of the mesophasic assembly.

158 It is known that GABA<sub>A</sub>R interacts with scaffolding molecules gephyrin and associated  
159 proteins that may interact with one another to form thin sheet-like densities in parallel to the  
160 postsynaptic membrane<sup>7</sup>. To examine whether such interactions might underlie the observed  
161 organization of GABA<sub>A</sub>Rs, we obtained a 2D density projection of the scaffolding layer (Fig.  
162 4a2). Distinct condensate-like densities were observed in the scaffolding layer (Fig. 4a2;  
163 Extended Data Fig. 7b), well correlated with the mesophasic assembly of GABA<sub>A</sub>Rs in  
164 majority of synapses (Fig. 4c). Furthermore, many particles in the scaffolding layer  
165 positioned directly underneath individual receptor densities, also with ~11 nm inter-particle  
166 distances (Fig. 4d). Quantitative analysis further confirmed that the density in the scaffolding  
167 layer was higher directly underneath a linked GABA<sub>A</sub>R within the phase boundary (Fig. 4e).  
168 In contrast, the higher peri-receptor scaffolding density was not observed for receptors  
169 outside the phase boundary, nor was it found for solitary receptors within the phase  
170 boundary, indicating that such receptors might not have direct interactions with the  
171 scaffolding molecules (Fig. 4e). Thus, the semi-ordered organization of linked receptors is  
172 likely due to their interaction with the underlying scaffolding molecules, which form a semi-  
173 ordered sheet-like condensate, probably through multivalent interactions.

174

175 **Mesophasic organization of PSD correlates with neurotransmitter release.** It is  
176 tempting to hypothesize that the mesophasic organization of GABA<sub>A</sub>Rs may functionally  
177 correlate with presynaptic neurotransmitter release. To test this, we analyzed the locations of  
178 synaptic vesicles near the presynaptic active zone. In our tomograms, two types of vesicles  
179 were identified: one tethered to the presynaptic membrane through rod-like densities, thus  
180 termed hereafter as tethered vesicles; the other had direct contact with the presynaptic  
181 membrane, thus termed as contacting vesicle (Fig. 4f). Both types of vesicle-plasma  
182 membrane interaction have also been observed in cryoET studies of purified  
183 synaptosomes<sup>36</sup>. Intriguingly, most (93%) of the contacting vesicles located within the



184 presynaptic area apposing to the postsynaptic region inside the phase boundary (Fig. 4f;  
185 Extended Data Fig. 7d). Outside the boundary, the number of contacting vesicles are  
186 significantly fewer as compared to that expected from random distribution. In contrast, the  
187 number of tethered vesicles located inside or outside this area are not significantly different  
188 from that of expected from random distribution (Fig. 4f; Extended Data Fig. 7d). It has been  
189 suggested that tethering allows initial targeting of vesicles to the membrane, and the  
190 contacting vesicles are more ready to release upon stimulation<sup>36,37</sup>. If this is the case, our  
191 observations suggest that vesicular GABA is primarily released towards the semi-ordered  
192 GABA<sub>A</sub>R networks within the mesophasic boundary, thus optimizing the efficiency of  
193 neurotransmission.

194

## 195 **Discussion**

196 By quantitative analysis of individual GABA<sub>A</sub>Rs in intact inhibitory synapses, our results  
197 reveal a unique mesophasic state of receptor assembly that is likely to result from the  
198 binding of these receptors to mutually-interacting scaffolding proteins such as gephyrin and  
199 their associated proteins<sup>38,39</sup>. Unlike the previous picture of a hexagonal lattice-shaped PSD  
200 architecture<sup>38,40,41</sup>, we observed semi-ordered receptor networks with a fixed value, 11-nm,  
201 for preferred inter-receptors distance, and with virtually uniformly distributed values for  
202 relative angles. This unique property suggests that the interaction among gephyrin  
203 molecules and associated proteins are also semi-ordered, forming flexible networks.  
204 Importantly, this network is confined to a two-dimensional sheet parallel to the postsynaptic  
205 membrane<sup>7</sup>, probably by the interaction of gephyrin with membrane-bound GABA<sub>A</sub>Rs. Such  
206 a mesophasic assembly exhibits both variability and regularity, demonstrating how  
207 ensembles of synaptic molecules acquire great complexity via self-organization. This  
208 organization principle may also suggest a molecular strategy for a synapse to achieve its  
209 “Goldilocks” state with a delicate balance between stability and flexibility on the micro-nano  
210 scale.

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310 **End notes**

311 **Supplementary information.** Supplementary Information is linked to the online version of  
312 the paper.

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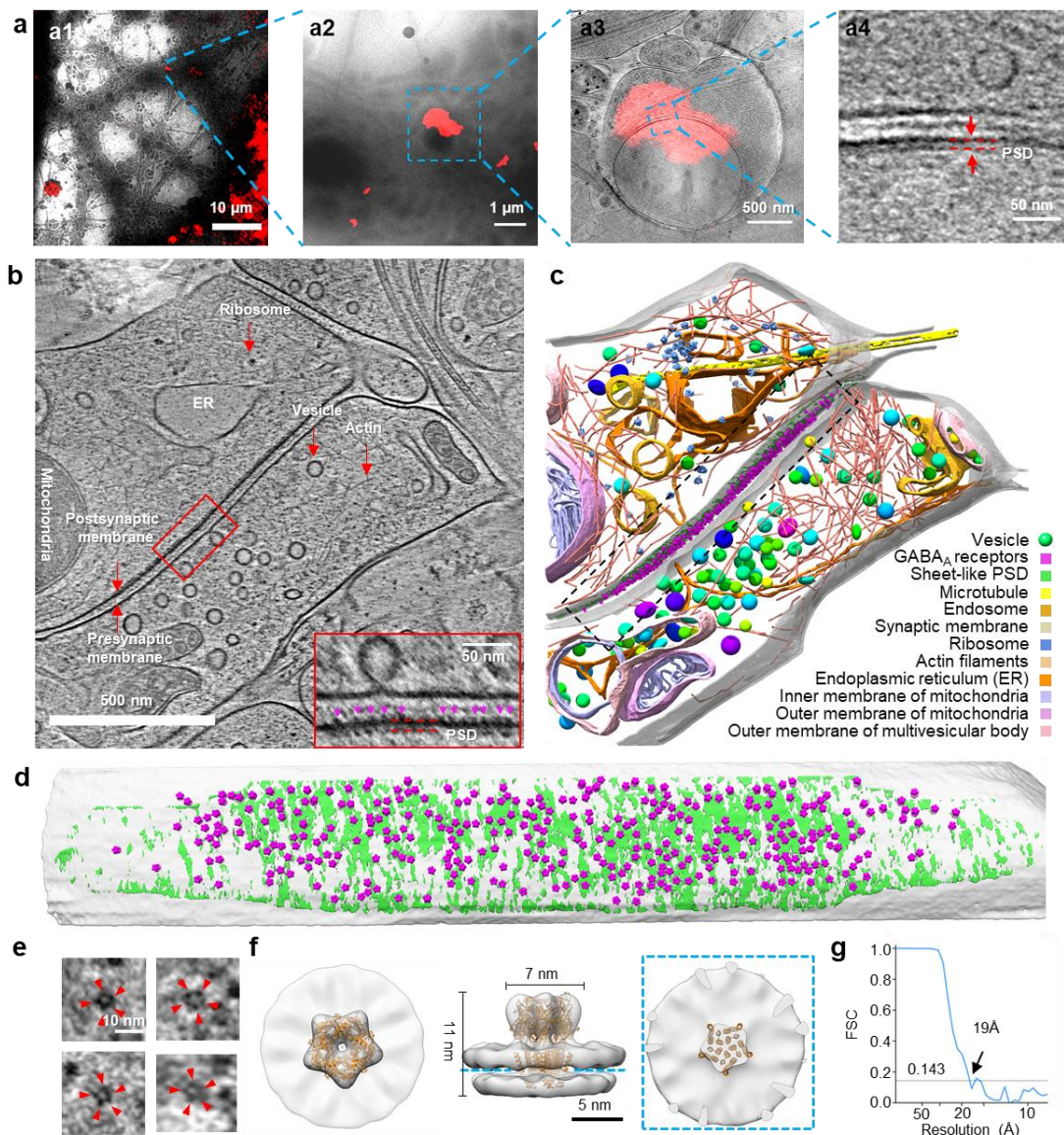
334 **Data and materials availability:** All data reported in the main text or the supplementary  
335 materials are available upon request.

336 **Code availability:** All code used in the paper is available upon request.



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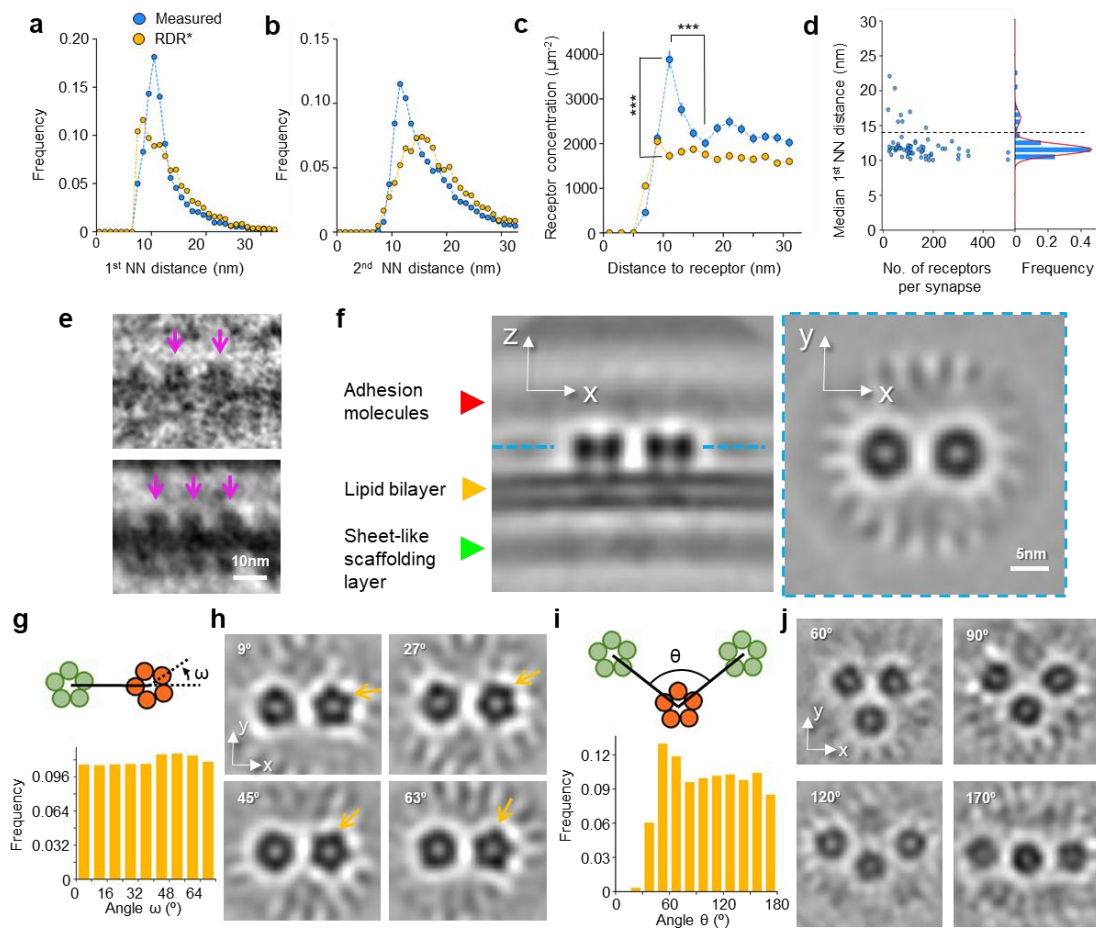
## Main figures and legends



338

339 **Fig. 1 | Identification and *in situ* structure of GABA<sub>A</sub>R in inhibitory synapse.** a,  
 340 Identification of inhibitory synapses with cryo-correlative light and electron microscopy. **a1**,  
 341 Low magnification EM image superposed with fluorescence image of gephyrin-mCherry. **a2**,  
 342 Zoomed-in view of (**a1**). **a3**, Electron tomographic slice superposed with fluorescence  
 343 puncta. **a4**, Zoomed-in view of (**a3**) showing thin sheet-like PSD. **b**, A tomographic slice of  
 344 an inhibitory synapse. Various subcellular components are labelled on the image. Inset:  
 345 Zoomed-in view showing receptor densities (magenta arrowheads). **c**, 3D rendering of the  
 346 tomogram shown in (**b**). **d**, Front view of GABA<sub>A</sub>Rs (purple), density of the scaffolding  
 347 protein layer (green) on the postsynaptic membrane (transparent gray). **e**, Example  
 348 tomographic slices of individual GABA<sub>A</sub>R in top view. Red arrowheads showing 5 blobs of

349 GABA<sub>A</sub>R density. **f**, Sub-tomogram average of GABA<sub>A</sub>R fitted with crystal structure (orange  
350 ribbons)<sup>22</sup>. **g**, Fourier shell correlation of the GABA<sub>A</sub>R sub-tomogram average.  
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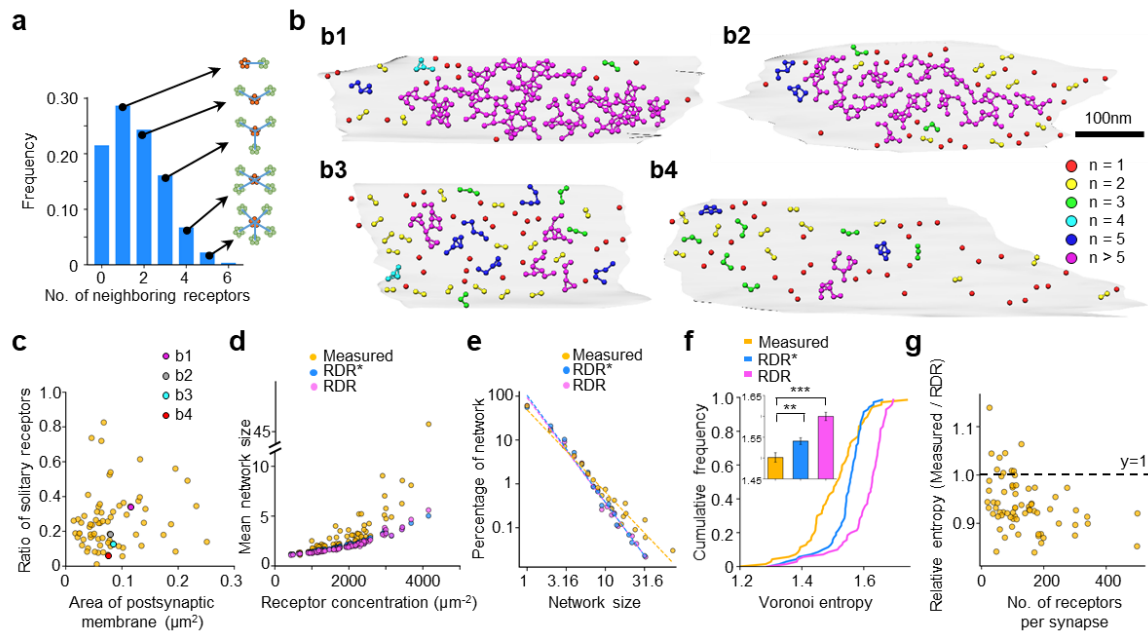
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**Fig. 2 | GABA<sub>A</sub>R super-complexes.** **a, b**, The first (**a**) and second (**b**) nearest neighbor (NN) distances distribution of measured receptors (Measured) and randomly distributed receptors without overlap (RDR\*, *i.e.* distance between any two receptors is larger than 7 nm).  $P < 0.001$ , K-S test in both (**a**) and (**b**). **c**, Receptor concentration as a function of distance to a GABA<sub>A</sub>R ( $n = 9,618$ ). \*\*\*,  $P < 0.001$ , two-tailed t-test. **d**, Left: Scatter plot of the number of receptors vs. the median first NN distance of each synapse. Right: Frequency distribution of median first NN distance, fitted with two Gaussian distributions (red curve). Dashed line shows the lowest point between two peaks. **e**, Examples of receptor pairs and triplets from original tomograms. Arrows point to receptors. **f**, Orthogonal slice views of the sub-tomogram average of receptor pairs. **g, i**, The distribution of relative rotation angles  $\omega$  (**g**) and  $\theta$  (**i**), as defined in respective diagrams. **h, j**, Sub-tomogram averages of receptor pairs with different  $\omega$  (**h**) and receptor triplets with different  $\theta$  (**j**). Error bars are SEM for all figures.

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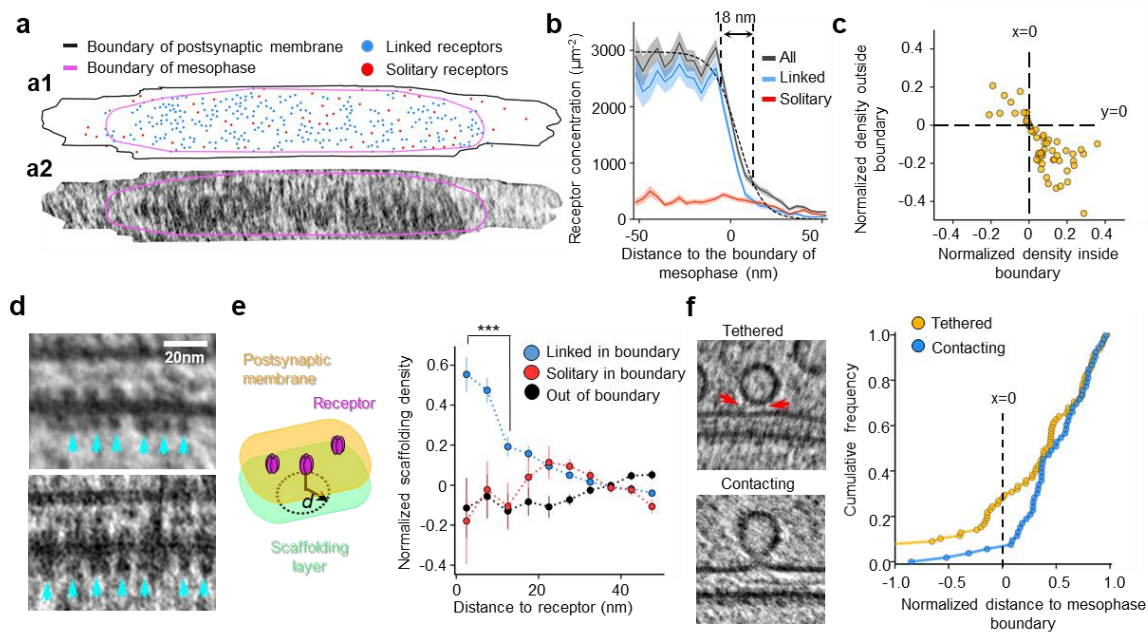


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369 **Fig. 3 | Two dimensional networks of GABA<sub>A</sub>Rs.** **a**, Distribution of the number receptors  
 370 with different numbers of 11-nm neighbors. **b**, Four examples (**b1-4**) of receptor network  
 371 organization on the postsynaptic membrane. Color indicates network size (n, the number of  
 372 receptor in a network). **c**, Scattered plot of the ratio of solitary receptors vs. the area of  
 373 postsynaptic membrane for each synapse. Colored dots (magenta, gray, cyan and red)  
 374 correspond to synapses in (**b**). **d**, Scattered plot of mean network size vs. receptor  
 375 concentration for measured receptors (Measured), randomly distributed receptors (RDR),  
 376 and randomly distributed receptors without overlap (RDR\*). **e**, Power law distribution of  
 377 network size in log-log plot. **f**, Cumulative frequency of Voronoi entropy for each synapse  
 378 (n=70). Inset: Mean value of Voronoi entropy. \*\*, P<0.01, \*\*\*, P<0.001, K-S test. **g**,  
 379 Scattered plot of relative entropy (defined as Measured/RDR) vs the number of receptors for  
 380 each synapse.

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**Fig. 4 | Mesophasic assembly of inhibitory PSD. a**, Examples of receptor distribution on the postsynaptic membrane (**a1**) and the corresponding density projection of the scaffolding layer (**a2**). **b**, Receptor concentration as a function of distance to the mesophase boundary (n=58). The dashed curve is a sigmoid function fitted with the black curve. Light shadow: SEM. Vertical dashed lines: 80%-20% width of the sigmoid function. **c**, Correlation of scaffolding layer density inside and outside mesophase. **d**, Example of interactions between receptors and scaffolding proteins. Cyan arrows indicate density of scaffolding proteins interacting with GABA<sub>A</sub>Rs. **e**, Left: Diagram showing relative positions of scaffolding layer and receptors, with  $d$  representing distance to the projection of receptor in scaffolding layer. Right: Normalized scaffolding density as a function of  $d$  (n=9,531). \*\*\*P<0.001, two-tailed t-test. **f**, Left: Example tomographic slices of tethered and contacting vesicles. Red arrows indicate rod-like tethers. Right: Cumulative frequency of normalized distance from vesicles to mesophase boundary. A vesicle with normalized distance of 1 means it is at the center of the mesophasic condensate, while a vesicle with normalized distance of 0 means it is on the mesophase boundary. N=81 for tethered vesicles; N=54 for contacting vesicles. The distributions of the two vesicle populations are significantly different (P=0.013, two-tailed t-test).

## 402 **Methods**

403 All animal experiments were approved by the Animal Experiments Committee at the  
404 University of Science and Technology of China.

### 406 **Primary culture of hippocampal neurons**

407 Low-density cultures of dissociated embryonic rat hippocampal neurons were prepared  
408 according to the protocols described previously<sup>7</sup>. In brief, electron microscopy (EM) gold  
409 finder grids (Quantifoil R2/2 Au NH<sub>2</sub> grids) were plasma cleaned with H<sub>2</sub> and O<sub>2</sub> for 10 s  
410 using a plasma cleaning system (Gatan), sterilized with UV light for 30 min, and then treated  
411 with poly-L-lysine before use. Hippocampi were dissected from embryonic day-18 rats and  
412 were treated with trypsin for 15 min at 37 °C. The dissociated cells were plated on the  
413 treated EM grids at a density of 40,000-60,000 cells/ml in 35-mm Petri-dish, and maintained  
414 in incubators at 37 °C in 5% CO<sub>2</sub> atmosphere. NeuroBasal (NB) medium (Invitrogen)  
415 supplemented with 5% heat-inactivated bovine calf serum (PAA), 5% heat-inactivated fetal  
416 bovine serum (HyClone), 1× Glutamax (Invitrogen) and 1× B27 (Invitrogen) was used as  
417 culture medium. Each Petri-dish was added with 1.5 ml medium. Twenty-four hours after  
418 plating, half of the medium was replaced with serum-free culture medium. Then, one-third of  
419 the culture medium was replaced with fresh serum-free culture medium every three days.  
420 The cultures were treated with cytosine arabinoside (Sigma) to prevent the overgrowth of glia  
421 cells. Some of the cultures experienced inactivation by 2-day treatment with 1 μM  
422 tetrodotoxin (TTX) or 1-hour treatment with 2 μM TTX followed by 3-hour treatment with 2  
423 μM TTX plus 50 μM APV<sup>42,43</sup>. We did not observe significant difference among different  
424 groups in basic properties of receptor expression and organization and thus pooled the data  
425 together for all analyses. For cryo correlative light and electron microscopy (cryoCLEM)  
426 experiments, cultures were transfected with mCherry-gephyrin (a gift from Dr. Ann Marie  
427 Craig) using Lentivirus at 10 DIV, as described previously<sup>7</sup>. All cultures were used for cryo-  
428 electron tomography (cryoET) imaging at 16 days *in vitro* (DIV).

### 430 **Frozen-hydrated sample preparation**

431 At DIV 16, the culture medium was replaced with extracellular solution (ECS, containing 150  
432 mM NaCl, 3 mM KCl, 3 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 10 mM HEPES and 5 mM glucose, pH 7.3).  
433 The EM grids were taken out from the CO<sub>2</sub> incubator and loaded into a Vitrobot IV (FEI)  
434 which was maintained in 100% humidity. Protein A-coated colloidal gold beads (15-nm size,  
435 CMC) were added to the grid (4 μl each, stock solution washed in ECS and diluted 10 times  
436 after centrifugation) as fiducial markers. The grids were then blotted and plunged into liquid  
437 ethane and were stored in liquid nitrogen until use.

438

### 439 **CryoCLEM imaging**

440 For cryoCLEM imaging, we used the same procedures in our previous paper<sup>7,44</sup>. In brief, the  
441 inside channel of the custom built cryo-chamber was precooled to -190 °C by liquid nitrogen,  
442 and maintained below -180 °C. Then, an EM grid with frozen-hydrated sample was loaded  
443 onto an EM cryo-holder (GATAN), which was subsequently inserted into the cryo-chamber.  
444 Dry nitrogen gas flowed around the 40X objective lens (Olympus LUCPLFLN 40×, NA 0.6)  
445 throughout the experiment to prevent the formation of frost. Fluorescence images were  
446 taken with an ANDOR NEO sCMOS camera (Andor) attached to the fluorescence  
447 microscope. For each field of view, both bright-field and mCherry channel (Ex: 562/40, DM:  
448 593, Em: 641/75; Semrock, mCherry-B-000) images were acquired.

449 The EM cryo-holder with the grid was then directly transferred into a Tecnai F20  
450 microscope (Thermo Fisher). Indexes of the finder grids were used to roughly identify the  
451 areas of the sample imaged in cryo-light microscope. Then *Midas* program in IMOD  
452 package<sup>45</sup> was used to roughly align the low magnification (330×) EM images with the bright-  
453 field LM images. After rough alignment, a set of holes (about 10 for each images) on the  
454 carbon film of the grid were manually picked using 3dmod in IMOD package from both the  
455 low magnification EM images and their corresponding fluorescence images. Transformation  
456 functions between the EM and LM images was calculated by correlating the selected  
457 positions in both images.

458 After aligning the low magnification EM images with LM images, pixel-wise positions of  
459 ~15 holes on carbon film (in one square) in each low magnification EM image were  
460 recorded. Afterwards, those holes were identified at 5,000× magnification and their  
461 mechanical coordinates (*i.e.* positions on the EM sample stage) were also recorded. The  
462 transformation function from the pixel-wise positions to EM mechanical coordinates was  
463 determined. Then the puncta of gephyrin-mCherry were selected manually using 3dmod in  
464 IMOD. Positions of these fluorescent puncta were then converted into corresponding EM  
465 mechanical coordinates with the transformation functions to guide tilt series acquisition.  
466 Finally, reconstructed tomographic slices were fine-aligned and merged with the  
467 fluorescence images to identify each synapse (Fig. 1a) using Midas and ImageJ.

468

### 469 **Cryo-electron tomography (CryoET) imaging**

470 For cryoCLEM experiments, the tilt series were collected using a Tecnai F20 microscope  
471 (Thermo Fisher) equipped with Eagle CCD camera (Thermo Fisher). The Tecnai F20 was  
472 operated at an acceleration voltage of 200 kV. Tilt series were collected first from 0° to -60°  
473 and then from +2° to +60° at 2° intervals using FEI Xplore 3D software, with the defocus  
474 value set at -12 to -18 μm, and the total electron dosage of about 100 e<sup>-</sup>/Å<sup>2</sup>. The final pixel

475 size was 0.755 nm.

476 For the analysis of GABA<sub>A</sub>Rs, cryoET data were collected using a Titan Krios (Thermo  
477 Fisher) equipped with a Volta phase plate (VPP), a post-column energy filter (Gatan image  
478 filter), and a K2 Summit direct electron detector (Gatan). The energy filter slit was set at 20  
479 eV. The Titan Krios was operated at an acceleration voltage of 300 KV. When VPP was  
480 used, defocus value was maintained at -1  $\mu\text{m}$ ; otherwise, it was maintained at -4  $\mu\text{m}$ . The  
481 VPP was conditioned by pre-irradiation for 60 s to achieve an initial phase shift of about  
482  $0.3\pi$ . Images were collected by the K2 camera in counting mode or super-resolution mode.  
483 When counting mode was used, the pixel size was 0.435 nm. For super-resolution mode  
484 image, the final pixel size was 0.265 nm. Tilt series were acquired using SerialEM<sup>46</sup> with two  
485 tilt schemes: from +48° to -60° and from +50° to +66° at an interval of 2°; from +48° to -60°  
486 and from +51° to +66° at an interval of 3°. The total accumulated dose is  $\sim 150 \text{ e}/\text{\AA}^2$ . For  
487 sub-tomogram analysis, 6 grids were used for data collection. Totally, 32 and 40 inhibitory  
488 synapses were imaged with and without VPP, respectively.

489

### 490 **3D reconstruction of the tomograms**

491 Each recorded movie stack was drift-corrected and averaged to produce a corresponding  
492 micrograph using MotionCorr<sup>47</sup>. To combine the data with different pixel size during image  
493 processing, we rescaled the images recorded with super-resolution mode with antialiasing  
494 filter to match the pixel size of image recorded with counting mode (0.435 nm/pixel), by  
495 *newstack* command in IMOD. For images recorded without VPP, the defocus value of each  
496 image was determined by CTFIND4<sup>48</sup>. For tilt series acquired with VPP, the defocus values  
497 cannot be precisely calculated. However, the defocus of each image is relatively low ( $\sim 1$   
498  $\mu\text{m}$ ), which does not limit the resolution obtained by sub-tomogram averaging. Thus, we did  
499 not perform defocus determination and CTF correction for these tilt series.

500 Tilt series were aligned with 15 nm gold beads as fiducial markers using IMOD. 3D  
501 reconstruction was performed with weighted back projection algorithm (WBP) using  
502 NovaCTF<sup>49</sup>. Because those tomograms had low contrast and were hard to interpret by visual  
503 inspection, we also used SIRT-like filter in NovaCTF to generate tomograms equivalent to  
504 those reconstructed by SIRT algorithm with 5 iterations. Segmentation and cryoET density  
505 analyses were performed using the SIRT-like filter reconstructed tomogram, whereas sub-  
506 tomogram averaging was performed using tomogram reconstructed with WBP.

507 Because the samples are thick, to eliminate the depth-of-the-focus problem, we  
508 performed 3D-CTF correction<sup>49</sup> and obtained CTF phase flipped tomograms for tilt series  
509 acquired without VPP. The defocus step for depth-of-the-focus correction was 50 nm.

510

### 511 **3D rendering**

512 By manually placing markers corresponding structures using *volume tracer* in UCSF  
513 Chimera<sup>50</sup>, synaptic membranes and organelles such as microtubules, actin filaments,  
514 mitochondria and multivesicular bodies were traced and segmented. Then, the manually  
515 segmented structures were smoothed by Gaussian filter. The ribosomes and synaptic  
516 vesicles were identified by template matching using PyTom<sup>51</sup>, as described previously<sup>7</sup>. The  
517 vesicles were rendered based on their diameter.

518

### 519 **Generating uniform oversampled points on postsynaptic membranes.**

520 Previous study showed the sub-tomogram average can be preformed with uniform selected  
521 sub-tomograms on a given surface taking advantage of the geometry of that surface<sup>52,53</sup>. We  
522 thus sought to reconstruct the structure of GABA<sub>A</sub>R by uniform oversampled sub-tomogram  
523 on the postsynaptic membrane segmented manually. Postsynaptic membrane was defined  
524 as the synaptic membrane area corresponding to the uniform synaptic cleft.

525 To segment postsynaptic membrane, we first segmented the synaptic cleft volumes in  
526 two-times binned tomograms using segmentation tool in Amira (Thermo Fisher). As the pixel  
527 size of all tomogram was or was scaled to 0.435 nm/pixel, the pixel size of two-times binned  
528 tomograms was 0.87 nm/pixel. Then we used Sobel filter to generate boundary surface of  
529 the segmented synaptic cleft. This boundary represented two opposed membranes:  
530 presynaptic and postsynaptic membrane. Then the postsynaptic membrane was manually  
531 extracted.

532 To generate uniformly oversampled points, we first generated a uniformly distributed 3D  
533 lattice of hexagonal close-packaging points in two-times binned tomograms (Extended Data  
534 Fig. 1a). The distance between the two nearest sampling points in the lattice is 5 pixels (4.35  
535 nm). All the sampling points were within 8.7 nm distance to the segmented membrane. The  
536 two-times binned sub-tomograms, whose centers are the sampling points, were then  
537 extracted using *boxstartend* program in IMOD. The extracted box size of each sub-  
538 tomogram is 32×32×32 pixels (27.84×27.84×27.84 nm). Because the sampling distance is 5  
539 pixels, the nearest distance from the center of any possible receptor to the one sampling  
540 point is less than 2.5 pixels. Given 7 nm (~8 pixels) diameter of GABA<sub>A</sub>R, each receptor  
541 should be fully covered in multiple extracted sub-tomograms so that no receptor was omitted  
542 during sampling.

543 The orientation of each sub-tomograms has three Euler angles denoted as parameters  
544 within the Relion star file<sup>25</sup>: rot (`_rlnAngleRot`), tilt (`_rlnAngleTilt`) and psi (`_rlnAnglePsi`).  
545 During the sub-tomogram extraction, the initial tilt and psi angles of each sub-tomogram  
546 were calculated as the orientation perpendicular to the patch of membrane in that sub-  
547 tomogram. The rot angle (rotational angle around the vector that is perpendicular to the  
548 membrane) for each sub-tomogram was set randomly.



549 With the uniform oversampling, we obtained 171,374 and 135,717 two-times binned  
550 sub-tomograms near postsynaptic membrane from tomograms imaged with and without  
551 VPP, respectively.

552  
553 **Initial 3D classification using two-times binned sub-tomograms.**

554 The classification and refinement of the sub-tomograms were performed using Relion  
555 (Extended Data Fig. 1b)<sup>25,54</sup>. The tomograms imaged with and without VPP appeared to be  
556 with different contrast. It is possible that 3D classification classifies the same protein feature  
557 into different classes based on whether the sub-tomogram was acquired with VPP or not. To  
558 minimize this error of classification, we performed the classification separately for sub-  
559 tomograms imaged with VPP and without VPP. This separation also enables cross-validation  
560 between results obtained from data acquired with VPP and without VPP (Extended Data Fig.  
561 1b).

562 To identify GABA<sub>A</sub>Rs containing tomograms from those sub-tomograms, we performed  
563 3D classification imposing 5-fold symmetry using Relion3. The resolution for the  
564 classifications was limited to 30 Å. To ensure that the orientation was searched around the  
565 vector perpendicular to membrane, we set the prior of tilt and psi angles as the calculated  
566 angles corresponding to the orientation of membrane and set the sigma of local angle  
567 search for tilt and psi angles as 3 degrees. We did not set any limitation in searching for the  
568 rot angle during classification. To limit the 3D positional search during 3D classification, the  
569 prior of the offset searching range was set as 0, meaning the offset was only searched  
570 around the center of the sub-tomograms. The offset search range was set to ±3 pixels. The  
571 initial reference was generated by *relion\_reconstruct* using the predetermined Euler angles.  
572 As expected, the initial reference appeared as a flat membrane structure due to the  
573 averaging of uniform oversampled sub-tomograms on the membrane (Extended Data Fig.  
574 1c). Because tilt series imaged without VPP were corrected using 3D-CTF and the tilt series  
575 imaged with VPP were recorded at low defocus value (-1 μm), we did not perform CTF  
576 correction during image processing using Relion. To compensate missing wedge, missing  
577 wedge volumes (`_lnCTFimage` in relion star file), which were 3D masks in Fourier space,  
578 were generated by custom made scripts. The classifications were performed with 100  
579 iterations (Extended Data Fig. 1c).

580 To determine the optimal number of classes for 3D classification, we tested the number  
581 of classes from 8 to 15 in classification. We obtained one 'good' class, which appeared  
582 similar to previously published GABA<sub>A</sub>R structures, for all number of classes from 8 to 13  
583 during the classification. The number of sub-tomograms in the 'good' class reduced as the  
584 number of classes increased from 8 to 11 but became stable after 11 (Extended Data Fig.  
585 2a). The structures of the classification result became worse when the number of class was

586 larger than 13. Thus, we used 12 as the optimal number of classes for classification and  
587 obtained the 'good' class among the 12 classes for both of the classifications using data  
588 collected with and without VPP (Extended Data Fig. 1b).

589 To eliminate that two or more sub-tomograms corresponding to the same receptor, we  
590 removed duplicated sub-tomograms as follows. We mapped the refined positions of the sub-  
591 tomograms after 3D classification to the original tomograms. If distances between centers of  
592 two classified sub-tomograms in original tomogram were smaller than 7 nm (the diameter of  
593 GABA<sub>A</sub>R), the sub-tomogram with lower score (`_rlnLogLikelihoodContribution` in Relion star  
594 file) were removed. After removing duplicate, we obtained 7,089 and 5,004 sub-tomograms  
595 obtained from data acquired with and without VPP, respectively.

### 596 597 **First round of 3D refinement using unbinned sub-tomograms.**

598 Then we calculated the coordinates of sub-tomograms in the corresponding unbinned  
599 original tomograms (with pixel size of 4.35 Å/pixel) and extracted new sub-tomograms with  
600 box size of 64×64×64 pixels. We combined sub-tomograms from VPP and no VPP data for  
601 3D auto-refine (Extended Data Fig. 1b). Then, we generated 60 Å resolution initial  
602 references by *relion\_reconstruct* with the predetermined orientations. Differing from the  
603 previous round of classification, we didn't limit the search angle and didn't set prior for angle  
604 and offset searching during the 3D refinement. Five-fold symmetry was imposed during 3D  
605 refinement. This round of 3D auto-refine refined the orientation and the positions of sub-  
606 tomograms and generated a preliminary reconstruction at 21 Å resolution, which was  
607 reported during *relion\_refine* processing. The duplicated sub-tomograms were further  
608 removed. After this step, we obtained 6,919 and 4,904 sub-tomograms for VPP and no VPP  
609 data, respectively.

### 610 611 **Removing outliers of tilt and psi angles.**

612 Because synaptic membrane is relatively flat and GABA<sub>A</sub>Rs are perpendicular to the  
613 membrane, the tilt and psi angles for sub-tomograms should be similar in each synapse.  
614 Thus, we used this knowledge to further reduce the error of receptor identification, as  
615 follows. We plotted the distributions of tilt and psi angles for the sub-tomograms in each  
616 synapse (Extended Data Figs. 2b-c). Indeed, the distribution of the refined tilt and psi angles  
617 of sub-tomograms in a given synapse were in a cluster with approximately Gaussian  
618 distribution, whose center corresponds to the angles perpendicular to the postsynaptic  
619 membrane (Extended Data Fig. 2c). Few sub-tomograms have orientations perpendicular to  
620 the membrane but pointing to the cytoplasmic side, possibly they were aligned to the  
621 proteins of postsynaptic densities (PSD) on the cytoplasmic side. We discarded those sub-  
622 tomograms for further refinement. The percentage of those misaligned sub-tomograms with

623 opposite orientation are 2% and 5% for VPP and no VPP data, respectively (Extended Data  
624 Fig. 1d). Furthermore, we also excluded sub-tomograms whose tilt and psi angles are three  
625 times of standard deviation ( $\sigma$ ) away from the center of the Gaussian distribution (10% and  
626 13% of total sub-tomograms from VPP and no VPP data, respectively) (Extended Data Figs.  
627 2c-e).

628

#### 629 **Removing outliers of low score.**

630 Then, we removed sub-tomograms with lower scores (`_rlnLogLikelihoodContribution` in  
631 Relion star file). We normalized the scores of sub-tomograms in each synapse, ensuring the  
632 normalized scores of the sub-tomograms for each synapse has an average of 0 and a  
633 standard deviation of 1. The distribution of normalized scores is a slightly lopsided Gaussian  
634 distribution (Extended Data Fig. 2f). We fitted the distribution with a Gaussian distribution  
635 and then removed the sub-tomograms with scores less than mean minus  $2\sigma$ . The ratio of  
636 sub-tomograms with lower score were  $\sim 3\%$  for both VPP and no VPP data (Extended Data  
637 Figs. 2e-g).

638

#### 639 **Second round of 3D refinement using unbinned sub-tomograms.**

640 After removing outliers, those sub-tomograms were used for a second round of 3D auto-  
641 refine (Extended Data Fig. 1b). Local searches with sigma angle of  $3^\circ$  for orientation  
642 determination were performed during 3D auto-refinement. Five-fold symmetry was imposed  
643 during 3D refinement. The final resolution of the reconstruction was estimated with two  
644 independently refined maps from halves of the dataset with gold-standard Fourier shell  
645 correlation (FSC) at the 0.143 criterion<sup>55</sup> using `relion_postprocess`, and was determined to  
646 be 19 Å (Fig. 1g).

647

#### 648 **Analysis the accuracy of rot angle.**

649 To estimate the accuracy of rot angle, we calculated two sets of cross-correlation (CC) score  
650 for the original sub-tomograms and sub-tomograms that rotated  $36^\circ$  (Extended Data Fig. 2h).  
651 CC score represents the similarity between a sub-tomogram and the sub-tomogram average  
652 of GABA<sub>A</sub>R. To do so, we rotated the sub-tomogram average by  $36^\circ$ , and then processed the  
653 sub-tomograms with `relion_refine` using original and rotated sub-tomogram averages as  
654 references, separately. We skipped both maximization step and alignment step in order to  
655 prevent updating references and orientation search, respectively. We used `always_cc`  
656 argument to calculate the CC score instead of log likelihood that was default in Relion. The  
657 processes were finalized with 1 interaction. By this processing, we obtained two new star  
658 files with the CC scores. We plotted the distribution of CC scores in the two star files.  
659 Indeed, the score distributions for the two sets of sub-tomograms are well separated



660 (Extended Data Figs. 2h-i).

661

### 662 **Estimate error rate of receptor identification**

663 To estimate the error of our 3D classification with uniformly over-sampled sub-tomograms,  
664 we visually inspected all the identified receptors in 4 selected tomograms acquired with VPP.  
665 Few receptors identified by our methods cannot be recognized, thus those receptors could  
666 be falsely identified. Thus, the error rate was defined as the percentage of identified receptor  
667 that cannot be recognized visually for each synapse.

668 The error rates for the 4 synapses are 14.4% (16 out of 111), 6.0% (5 out of 83), 22.9%  
669 (32 out of 140), and 18.3% (62 out of 339), respectively.

670

### 671 **False positive rate of receptor identification**

672 In order to evaluate the false positive rate of GABA<sub>A</sub>R identification, we repeated the sub-  
673 tomogram analysis using data mixing the same sub-tomograms and intentionally induced  
674 negative controlled sub-tomograms on presynaptic membrane (Extended Data Fig. 3).

675 These negative controlled sub-tomograms were extracted using the same uniform  
676 oversampling methods on the segmented presynaptic membranes. Presynaptic membranes  
677 of 2 inhibitory synapses imaged with VPP and 2 inhibitory synapses imaged without VPP  
678 were used for this analysis.

679 We did the classifications and refinements (Extended Data Figs. 3a-b) exactly the same  
680 as the previous described steps. The classifications and refinements with data mixing with  
681 negative controlled sub-tomograms also generated structures of GABA<sub>A</sub>Rs. As expected, the  
682 number of GABA<sub>A</sub>Rs identified using sub-tomograms with negative controlled sub-  
683 tomograms for each synapse is similar to the receptor identified without negative controlled  
684 sub-tomograms (Extended Data Fig. 3c).

685 For synapses analyzed for both pre- and post- synaptic membrane, we calculated false  
686 positive rate as falsely identified receptors on presynaptic membranes dividing number of  
687 receptors on post synaptic membranes. The false positive rates for the two synapses  
688 imaged with VPP are 15% and 10%. The false positive rates for the two synapses imaged  
689 without VPP are 13% and 10% (Extended Data Fig. 3d).

690

### 691 **3D classification of the oversampled sub-tomograms without symmetry**

692 We then tested whether the classification without symmetry could yield structures similar to  
693 the GABA<sub>A</sub>R structure published before. We used the same sub-tomograms acquired with  
694 VPP and performed the classification without symmetry. The other parameters were the  
695 same as the first round classification described before. Indeed, this classification generated  
696 structures with sizes similar to the GABA<sub>A</sub>R. However, the structures were worse than the

697 reconstruction with 5-fold symmetry and were not centered properly (Extended Data Fig. 4a).  
698 Intriguingly, two receptor-like structures could present in the same sub-tomogram average  
699 (Extended Data Fig. 4b). This further confirmed that the receptors tend to form receptor pairs  
700 with 11 nm inter-receptor distance.

701

### 702 **Analysis and reconstruction of receptor pair**

703 For each receptor pair (with  $11 \pm 4$  nm inter-particles distance), we calculated the coordinate  
704 of the center of the two GABA<sub>A</sub>Rs, and used this coordinate to extract sub-tomograms  
705 ( $64 \times 64 \times 64$  pixels) in two-times binned original tomograms. The tilt and psi angles of a  
706 receptor pair was set as the mean of those angles for the two receptors. The rot angle was  
707 calculated to ensure that the vector from one receptor to the other receptor aligns to the x  
708 axis of the receptor pair reconstruction (Fig. 2f). We then reconstructed receptor pair using  
709 *reliion\_reconstruct* with the calculated orientations. Total 15,184 sub-tomograms of receptor  
710 pairs were used in the reconstruction.

711

### 712 **Measuring the angle ( $\omega$ ) between the rotation of the receptor and pair axis**

713 We then calculated the angle ( $\omega$ ) between the rotation angle (rot) of one given receptor in a  
714 receptor pair to receptor pair axis (Fig. 2g). The receptor pair axis was defined as a vector  
715 from the other receptor to the given receptor. Then we separated the sub-tomograms into  
716 four groups by the  $\omega$  angle—  $0-18^\circ$ ,  $18-36^\circ$ ,  $36-54^\circ$ ,  $54-72^\circ$  groups, containing 3,883; 2,957;  
717 4,199 and 4,195 sub-tomograms, respectively. We further reconstructed the sub-tomograms  
718 in each group using *reliion\_reconstruct*. In all four reconstructions, the given receptor  
719 appears to have pseudo 5-fold symmetry.

720

### 721 **Reconstructing receptor triplet and analyzing the angle $\theta$ between the two arms.**

722 One GABA<sub>A</sub>R can also pair with two neighbor receptors forming receptor triplet. Each triplet  
723 has two arms, which connect the central receptor to the two neighbor receptors. We then  
724 calculated the angle  $\theta$  between the two arms of the triplet (Fig. 2i). We reconstructed the  
725 triplets with  $50-70^\circ$ ,  $80-100^\circ$ ,  $110-130^\circ$ ,  $160-180^\circ$  of  $\theta$  value, containing 2,428; 1,772; 1,937  
726 and 1,714 sub-tomograms, respectively (Fig. 2j). The center of each receptor triplet sub-  
727 tomogram was set as the mass center of the three receptors. Tilt and psi angles of each sub-  
728 tomogram were set as the mean angles of the three receptors. The rot angle of a receptor  
729 triplet sub-tomogram was calculated to ensure that the vector from one neighbor receptor to  
730 the other is parallel to x axis. All reconstructions were computed using *reliion\_reconstruct* by  
731 the sub-tomograms ( $64 \times 64 \times 64$  pixels) extracted from two-times binned tomograms.

732

### 733 **Local receptor concentration and nearest neighbor distance analysis**

734 Among the 72 synapses we obtained, 2 of them imaged without VPP were not fully covered  
735 in the tomograms. These two synapses were excluded in the analyses of GABA<sub>A</sub>Rs  
736 distribution in the following sections.

737 We calculated the concentration of receptors around a given point on the membrane. In  
738 our case, the given point is either a receptor or random selected point on postsynaptic  
739 membrane. We partitioned the membrane around the given point into concentric rings of 2  
740 nm width. The radius range of the rings is from 0 to 32 nm. Then, the receptor concentration  
741 was calculated as number of receptors in a ring dividing the surface area of that ring.

742 We also calculated the first and second nearest neighbor distance for each receptor,  
743 using standard distance formula in 3D.

744

### 745 **Analysis of the receptor networks**

746 If two receptors have distance smaller than 15 nm, they were defined as “linked” receptors.  
747 We then defined a receptor network as follows. If two receptors are linked by series of (equal  
748 or more than 0) receptors, we grouped them in the same network. Otherwise, they are in  
749 different networks. The network size was defined as number of receptors in a network.  
750 Randomized receptor distributions were generated from the same number of receptors over  
751 the same postsynaptic area.

752

### 753 **Calculation of the Voronoi entropy**

754 To calculate the Voronoi entropy of each synapse, we first calculated the first two principle  
755 vectors for all 3D segmented points on postsynaptic membrane, using singular value  
756 decomposition in Matlab. Using the two principle vectors, we projected the 3D receptor  
757 locations on a 2D plane. Then we generated Voronoi tessellation of the 2D locations of  
758 receptors in each synapse (Extended Data Fig. 6c) using `scipy.spatial.Voronoi` function in  
759 SciPy (<https://scipy.org>). Voronoi entropy was calculated using follow formula<sup>32</sup>:

760

$$V = - \sum_i p_i \ln(p_i)$$

761 Where  $i$  is the number of vertices of a polygon.  $p_i$  is the frequency of the polygon with  $i$   
762 vertices.  $\ln$  is the natural logarithm.  $V$  is the Voronoi entropy.

763

### 764 **Determining the boundary of mesophasic assembly of GABA<sub>A</sub>Rs**

765 To determine the boundary of the receptor assembly, the receptor positions were first  
766 projected onto the 2D plane as described before. Then, a convex hull of all linked receptors  
767 for each synapse were constructed using python package *shapely*  
768 (<https://github.com/Toblerity/Shapely>). To eliminated the coincidentally formed linked receptors  
769 outside the condensed receptors region, we smoothed the convex hull by 40 nm dilation

770 followed by 40 nm erosion using python package shapely (Extended Data Fig. 7a). Convex  
771 hull of 12 (out of 70) synapses have diameter smaller than 80 nm. Those synapses are not  
772 eligible for dilation, so they were excluded in the phase boundary analysis. The distance of a  
773 receptor to mesophase boundary was also calculated using shapely.

774

#### 775 **Calculation of synaptic membrane area**

776 To calculate the area of postsynaptic membrane, we first generated surface of the  
777 postsynaptic membrane in 3D using *imodmesh* in IMOD. The area of postsynaptic  
778 membrane was extracted from output of *imodinfo* command in IMOD. While in Fig. 4b and  
779 Extended Data Fig. 7c, the postsynaptic membranes were projected to a two dimensional  
780 plane. Thus in those figures, membrane areas were calculated two dimensionally using  
781 *shapely*.

782

#### 783 **Analysis electron microscopy density of scaffolding layer**

784 To analysis the density of scaffolding layer, we first extracted the voxels in the scaffolding  
785 layer region in the tomogram as densities 10-15 nm toward the cytoplasmic side from the  
786 postsynaptic membrane. The scaffolding layer region were then project to a 2D plane, using  
787 two principle vectors of postsynaptic membrane described previously, resulting in 2D density  
788 profiles of the scaffolding layer parallel to the postsynaptic membrane. The 2D density  
789 profiles were then normalized so that the mean pixel density of the profiles is 0 and the  
790 standard deviation is 1. The mesophase boundary of GABA<sub>A</sub>R were mapped on the 2D  
791 profile of the scaffolding layer. Densities inside and outside mesophase boundary on the 2D  
792 profiles were calculated as the mean pixel density inside and outside of the boundary,  
793 respectively.

794 We then calculated the density of scaffolding layer around 2D projected locations of  
795 receptors (Fig. 4e). We partitioned the 2D profile of scaffolding layer around a receptor into  
796 concentric rings of 5 nm width. The radius range of the rings is from 0 to 50 nm. Then, the  
797 density of scaffolding layer was calculated as the mean intensity value both in the concentric  
798 ring and inside postsynaptic membrane area (Fig. 4a). Hence, we produced the relation  
799 between the distance to the given receptor and the pixel density values of the 2D profile of  
800 scaffolding layer.

801

#### 802 **Analysis of tethered and contacting synaptic vesicles**

803 To calculate distance from synaptic vesicle to mesophase boundary, we first manually  
804 selected positions on the presynaptic membrane nearest to a contacting or a tethered  
805 vesicle. Then, the positions were projected to 2D plane using the methods described before.  
806 The distance ( $d_1$ ) from the synaptic vesicle projection point to mesophase boundary were

807 calculated using *shapely*. We also project all segmented points inside mesophase on  
808 postsynaptic membrane to the 2D plane and calculated the largest distance ( $d_2$ ) from those  
809 points to the mesophase boundary. The normalized distance from synaptic vesicle to  
810 mesophase boundary was calculated as  $d_1/d_2$ . Randomized vesicles were generated by  
811 randomly selecting locations over the same synaptic area. We repeated the randomization  
812 10 times for each synapses. The mean number of randomized vesicles inside or outside of  
813 mesophase boundary were used for statistical analysis.

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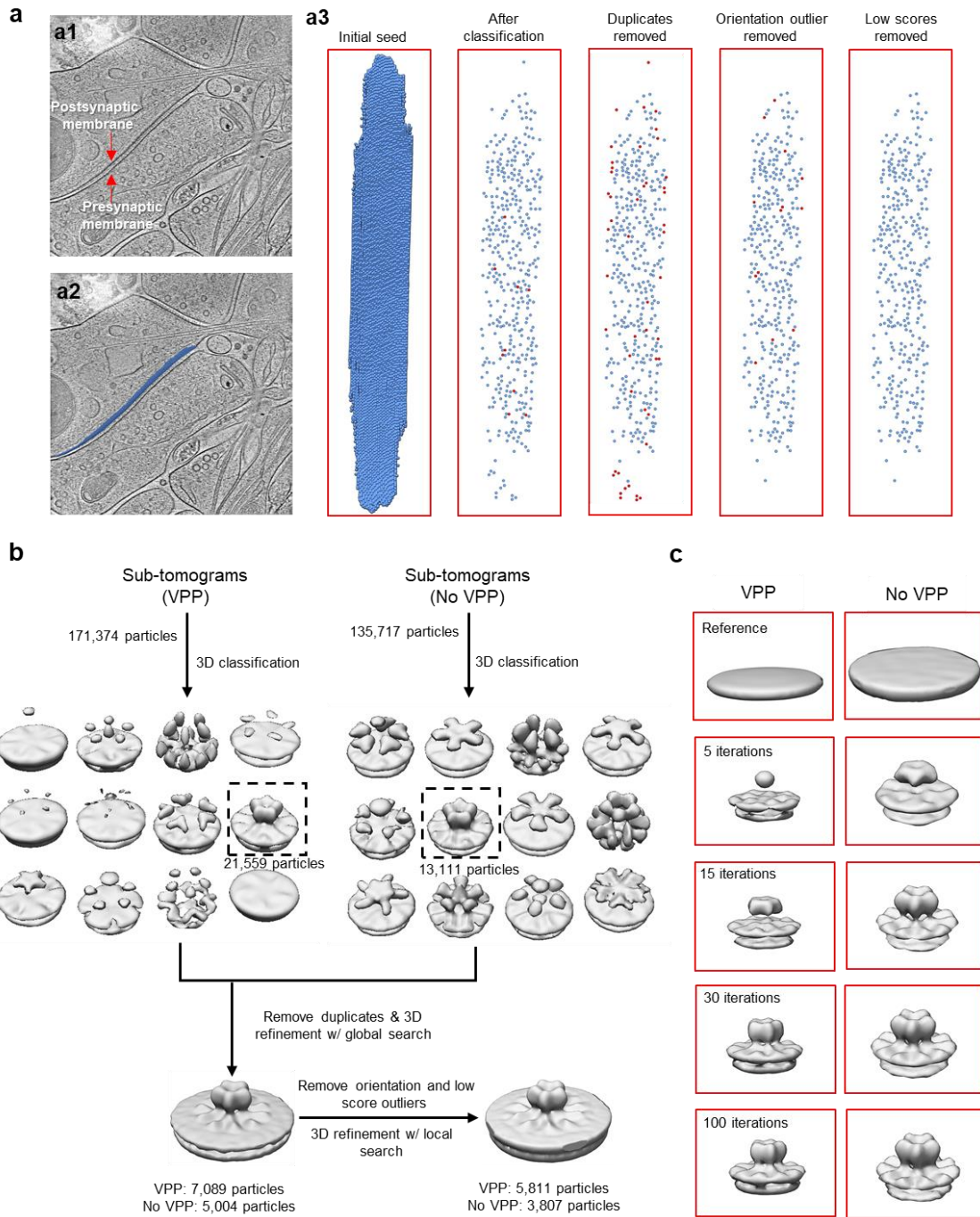
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## Extended data figures, table, and legends



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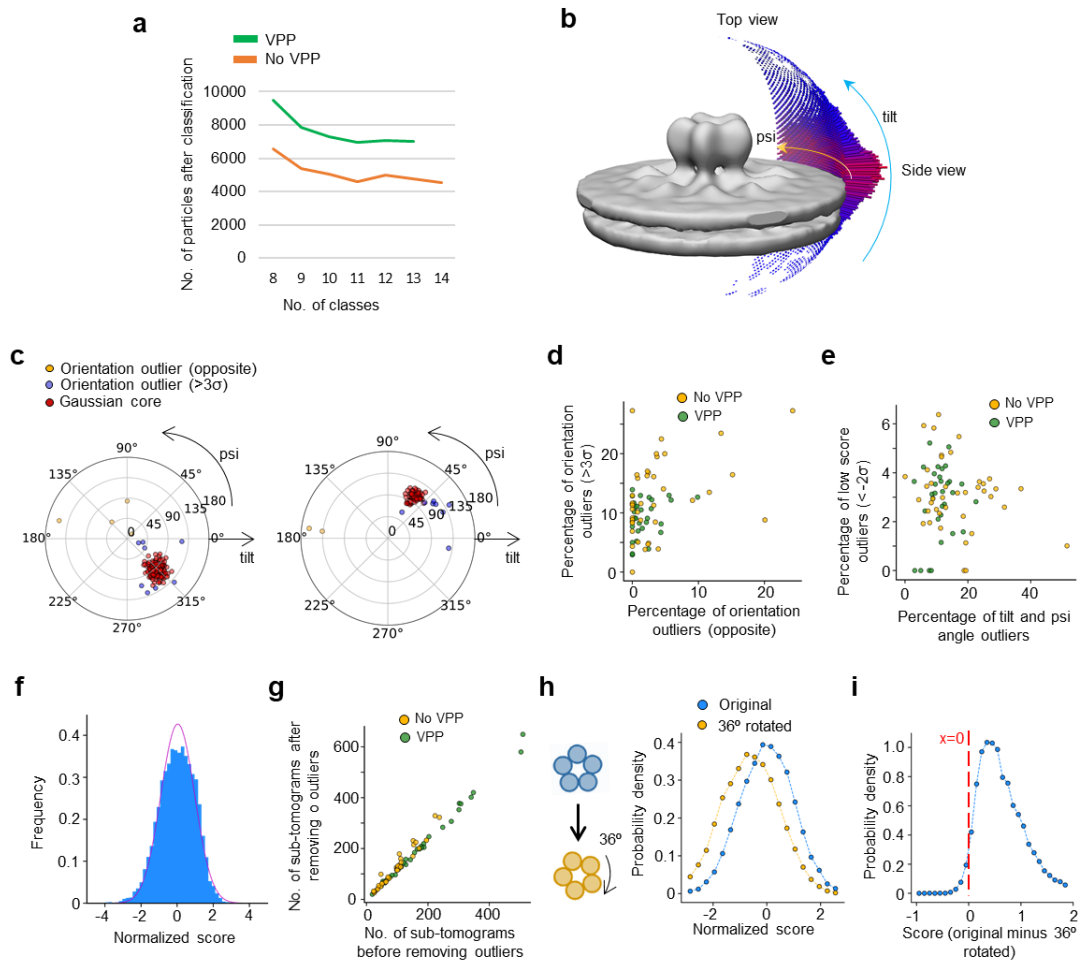
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**Extended Data Fig. 1 | Flowchart illustrates identification and sub-tomogram averaging of GABA<sub>A</sub>R.** **a**, Steps for identifying GABA<sub>A</sub>R. **a1**, Electron tomographic slice of an inhibitory synapse. **a2**, Electron tomographic slice superposed with sampling points on postsynaptic membrane. **a3**, Sampling points after each step. Red points are sampling points that will be discarded in the next step. **b**, Classification

859 and refinement of GABA<sub>A</sub>R on postsynaptic membrane. **c**, Structure of GABA<sub>A</sub>R emerged  
860 during iterative classification.  
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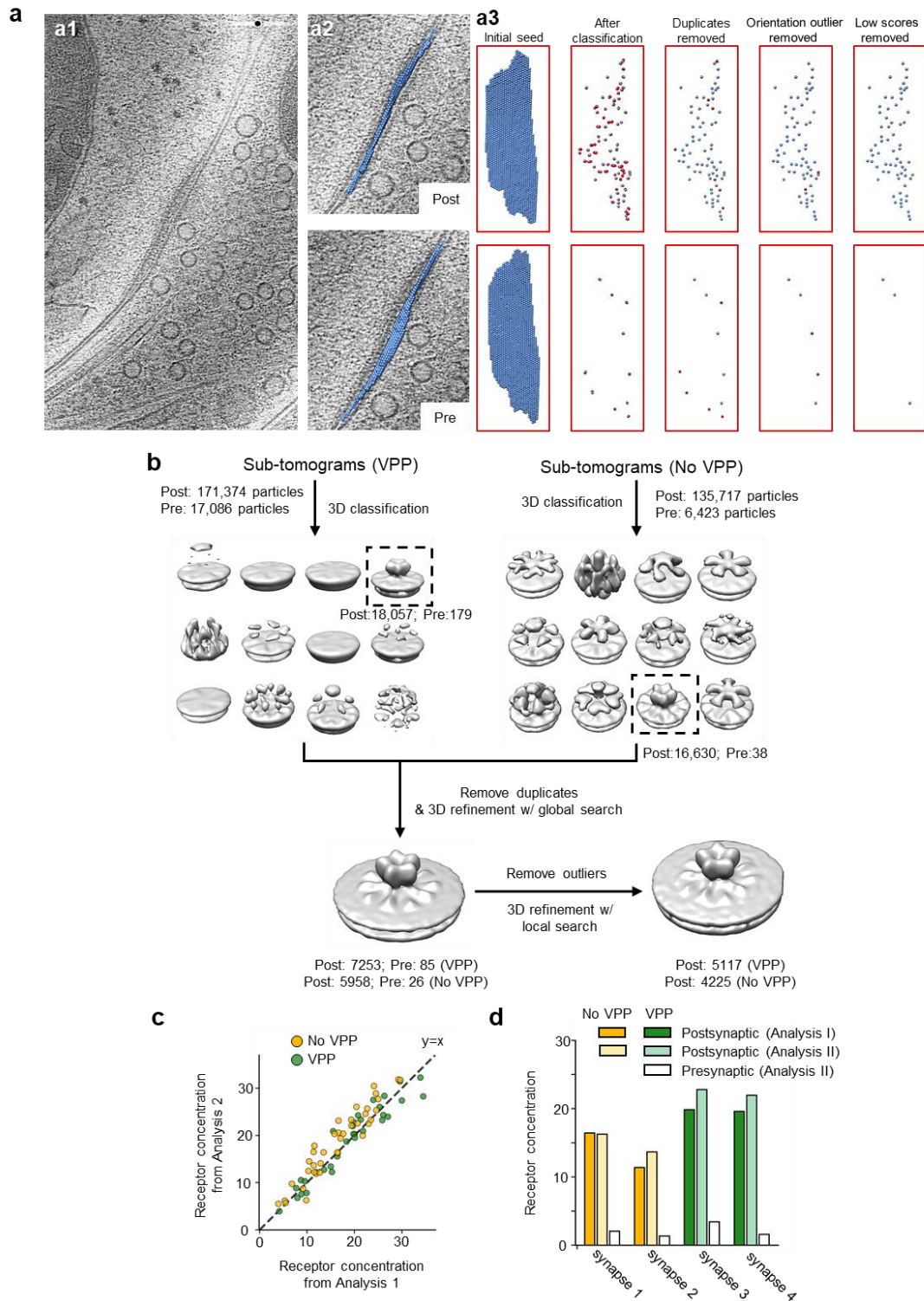
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### Extended Data Fig. 2 | Performance estimation of template-free classification and

**refinement.** **a**, Number of classified GABA<sub>A</sub>R sub-tomograms plotted against number of classes. **b**, Euler (psi and tilt) angles of all sub-tomograms used for the final sub-tomogram averaging. **c**, Distribution of Euler angles for sub-tomograms in two example synapses after first round of refinement. **d**, Percentage of outliers with opposite angles verses percentage of outliers with angles  $3\sigma$  away from Gaussian core in each synapse. **e**, Percentage of all orientation outliers verses percentage of low score outliers in each synapse. **f**, Frequency distribution of sub-tomogram scores fitted with Gaussian curve (red curve). **g**, Number of sub-tomograms before and after removing both orientation and low score outliers. **h**, Normalized CC score distribution of sub-tomograms comparing with original and  $36^\circ$  rotated sub-tomogram averages. **i**, Distribution of CC score differences for sub-tomograms comparing with original and  $36^\circ$  rotated sub-tomogram averages.





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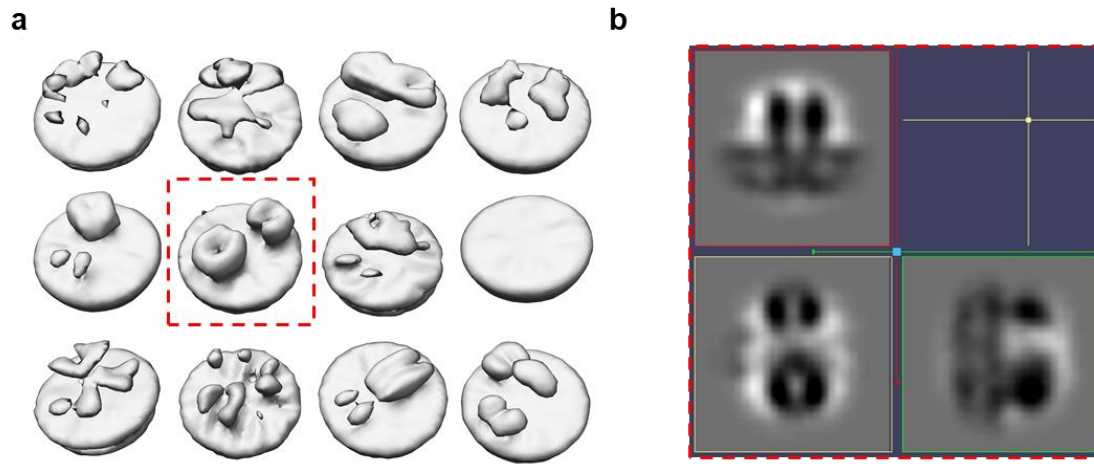
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**Extended Data Fig. 3 | Identification of GABA<sub>A</sub>Rs using sub-tomograms mixed with sub-tomograms on presynaptic membrane.** **a**, Steps for identifying GABA<sub>A</sub>Rs from sampling points mixed with sub-tomograms on presynaptic membrane. **a1**, Electron tomographic slice of an inhibitory synapse. **a2**, Electron tomographic slices superposed with sampling points on postsynaptic membrane (top) or presynaptic membrane (bottom). **a3**,

882 Sub-tomogram sampling points on postsynaptic membrane (top) or presynaptic (bottom)  
883 after each step. Red points are sampling points that will be discarded in the next step. **b**, 3D  
884 classification and refinement of sub-tomograms on 72 postsynaptic membranes and 4  
885 presynaptic membranes. **c**, Receptor concentration from Analysis II (analysis of sub-  
886 tomograms mixed with presynaptic sub-tomograms) verses receptor concentration from  
887 Analysis I (analysis of sub-tomograms without presynaptic sub-tomograms). **d**,  
888 Concentration of identified receptors on postsynaptic membranes in Analysis I, Analysis II,  
889 and falsely identified receptors on presynaptic membranes in Analysis 2 for the 4 selected  
890 synapses.

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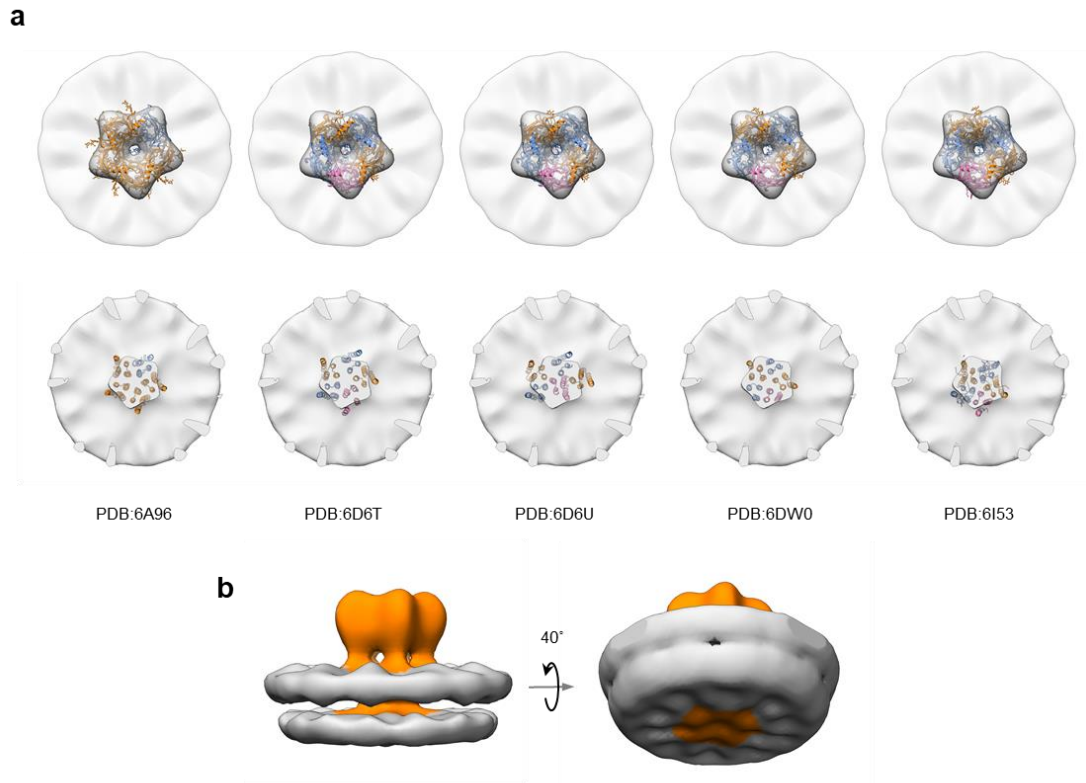
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**Extended Data Fig. 4 | Classification of oversampled sub-tomograms without symmetry.** **a**, Structures obtained from the 3D classification with VPP data. **b**, Orthogonal slice views of the structure boxed in (a).



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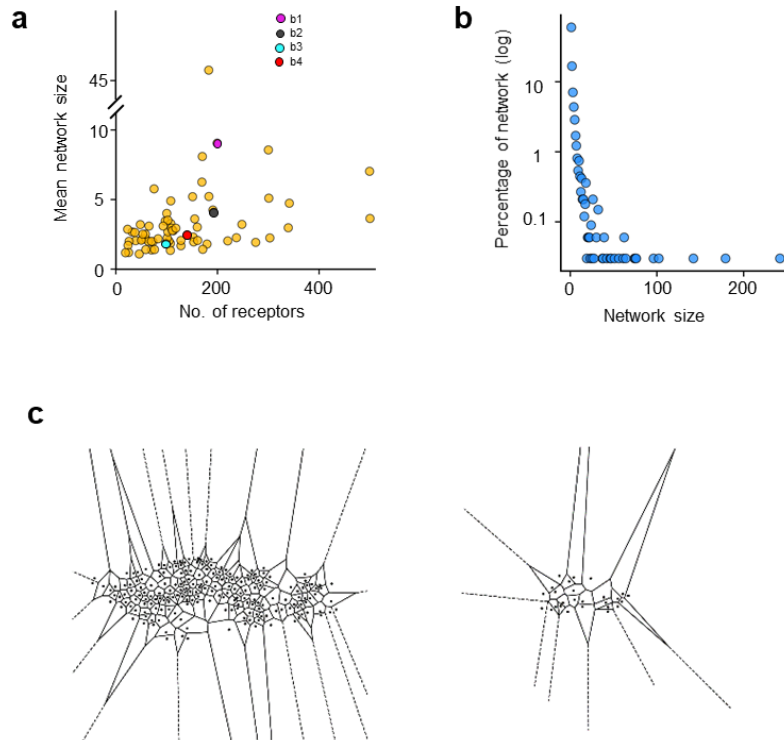
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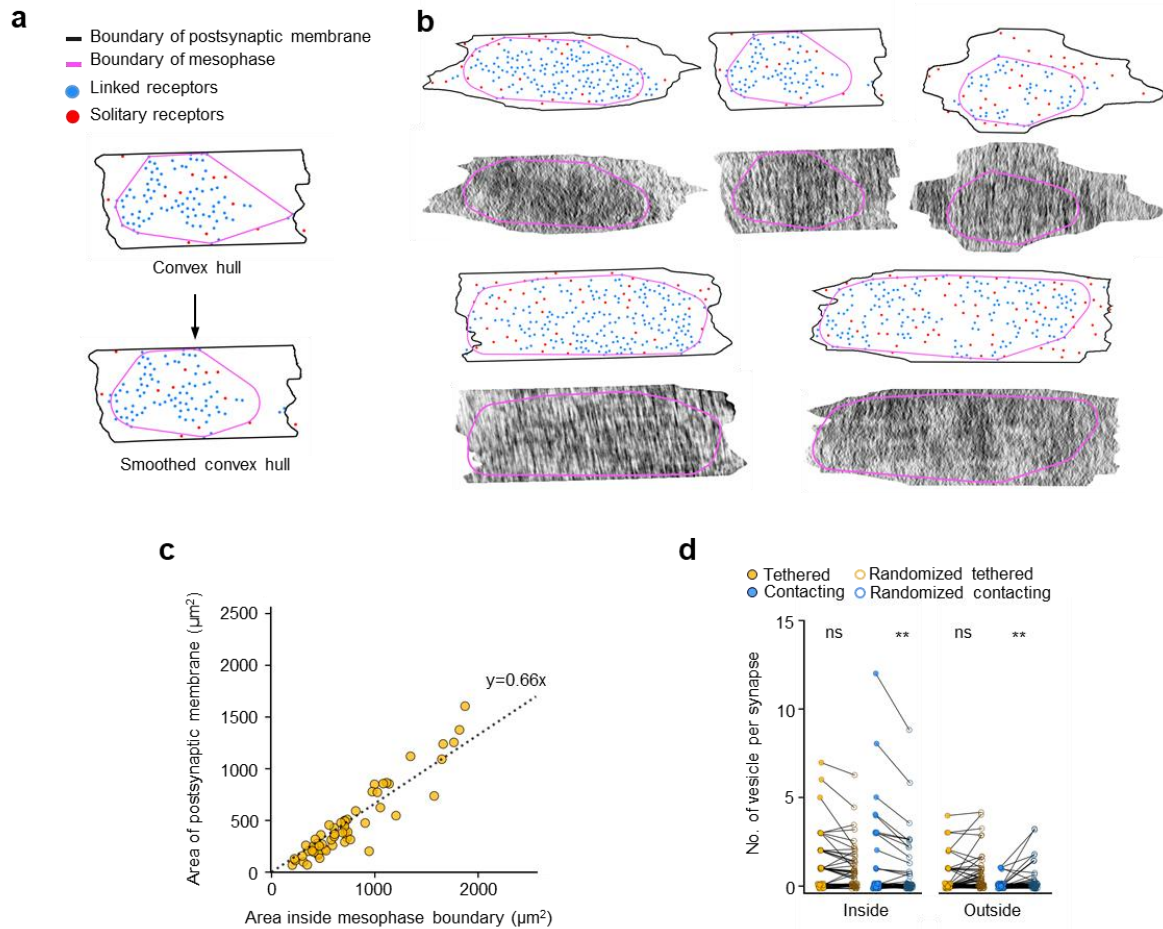
**Extended Data Fig. 5 | Structure features of sub-tomogram average of GABA<sub>A</sub>R. a,** Density of the sub-tomogram average fitted with atomic models of different subunit compositions or conformations<sup>26-29</sup>. **b, Left:** Sub-tomogram average of GABA<sub>A</sub>R. Orange density is GABA<sub>A</sub>R density. Gray density is membrane bilayer. **Right:** Rotated view of sub-tomogram average of GABA<sub>A</sub>R displayed at low threshold.



904

905 **Extended Data Fig. 6 | Two dimensional networks of GABA<sub>A</sub>Rs.** **a**, Scattered plot of  
906 mean network size (number of receptors divided by number of networks) and number of  
907 receptors for each synapse. Colored dots (magenta, gray, cyan and red) correspond to the  
908 four synapses in Fig. 3b respectively. **b**, Distribution of network size. Y-axis is plot in  
909 logarithm scale. **c**, Examples of Voronoi tessellation of receptors on postsynaptic membrane.  
910 Black dots represent the localizations of GABA<sub>A</sub>Rs.

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913 **Extended Data Fig. 7 | Mesophasic assembly of PSD. a**, Example of convex hull and  
 914 smooth convex hull of linked receptors. **b**, Examples of receptors distributions and 2D  
 915 density profiles of scaffolding layer. **c**, Scatted plot of area inside mesophase boundary and  
 916 area of postsynaptic membrane, fitted with a dashed line. **d**, Comparison between the  
 917 number of either type of vesicles inside or outside of mesophase boundary with the  
 918 corresponding number based on randomized vesicle distribution within the whole synapse  
 919 ( $n=58$  synapses). ns,  $P=0.20$ ; \*\*,  $P=0.005$ , paired two-tailed t-test.



920

### Extended Data Table 1 | Proteomes on inhibitory postsynaptic membrane

Protein name	Uniprot ID	Expt2 115/116 *	Transmembrane helices	Functional classification
Gamma-aminobutyric acid receptor subunit gamma-2	P18508	1.314	4	GABA <sub>A</sub> R subunits
Neuroigin-2	Q62888	1.247	1	Adhesion molecules
Neuroigin-1	Q62765	1.129	1	Adhesion molecules
Gamma-aminobutyric acid receptor subunit beta-1	P15431	1.077	4	GABA <sub>A</sub> R subunits
Gamma-aminobutyric acid receptor subunit beta-3	F1LRQ0	1.041	4	GABA <sub>A</sub> R subunits
Gamma-aminobutyric acid receptor subunit beta-2	P63138	0.984	4	GABA <sub>A</sub> R subunits
MAM domain-containing glycosylphosphatidylinositol anchor protein 2	P60756	0.965	1	Extracellular matrix proteins
Gamma-aminobutyric acid receptor subunit alpha-2	F1LMU7	0.842	4	GABA <sub>A</sub> R subunits
Gamma-aminobutyric acid receptor subunit alpha-3	F1LNZ5	0.828	4	GABA <sub>A</sub> R subunits
Gamma-aminobutyric acid receptor subunit alpha-1	P62813	0.56	4	GABA <sub>A</sub> R subunits
Protein Cdh10	F1LR98	0.539	1	Adhesion molecules
Protein Eln1	D3ZZ44	0.507	1	Other receptors
Receptor tyrosine-protein kinase erbB-4 (Fragment)	E9PTH1	0.461	2	Other receptors
Protein Igsf9b	D3ZB51	0.398	1	Adhesion molecules
Protein Slitrk2	D3ZK41	0.301	1	Adhesion molecules
Neurogenic locus notch homolog protein 1	Q07008	0.268	0	Other receptors
Gamma-aminobutyric acid type B receptor subunit 1	Q9Z0U4	0.23	8	GPCR
Protein Slitrk3	D4A062	0.205	2	Adhesion molecules
Protein Thsd7a	F1MA97	0.181	1	Extracellular matrix proteins
Netrin receptor DCC	Q63155	0.12	2	Other receptors
Teneurin-2	Q9R1K2	0.12	1	Other receptors
Isoform 2 of Teneurin-2	Q9R1K2-2	0.108	1	Other receptors
Protein Flrt2	D3ZTV3	0.104	1	Adhesion molecules

921 \*: Rank of biotinylation by synaptic cleft-target horseradish peroxidase (HRP) / cell surface

922 target HRP.

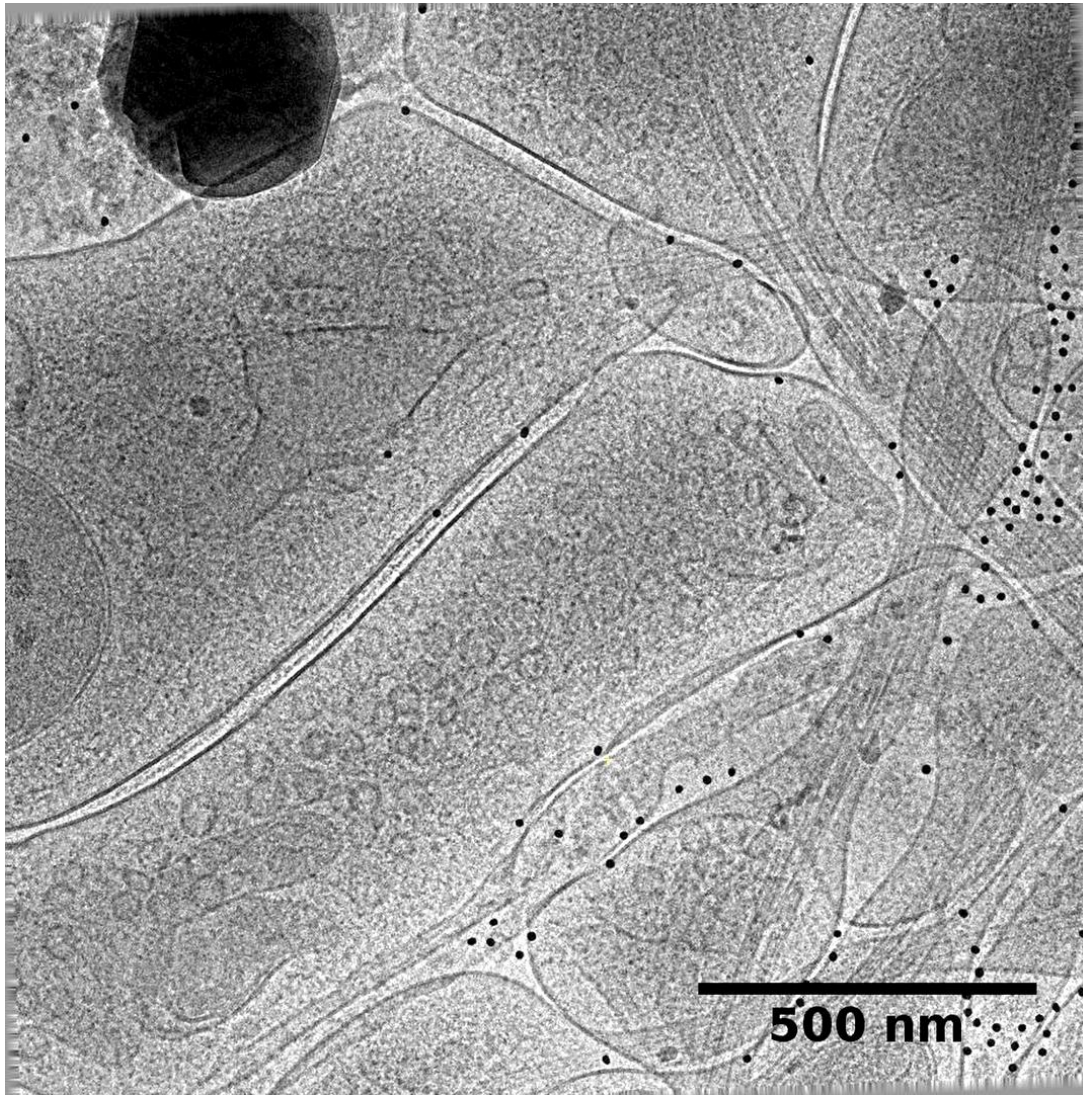
923 GABA<sub>A</sub>R subunits are shown in red.

924 All data from Loh et.al 2016<sup>23</sup>.

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## Supplementary



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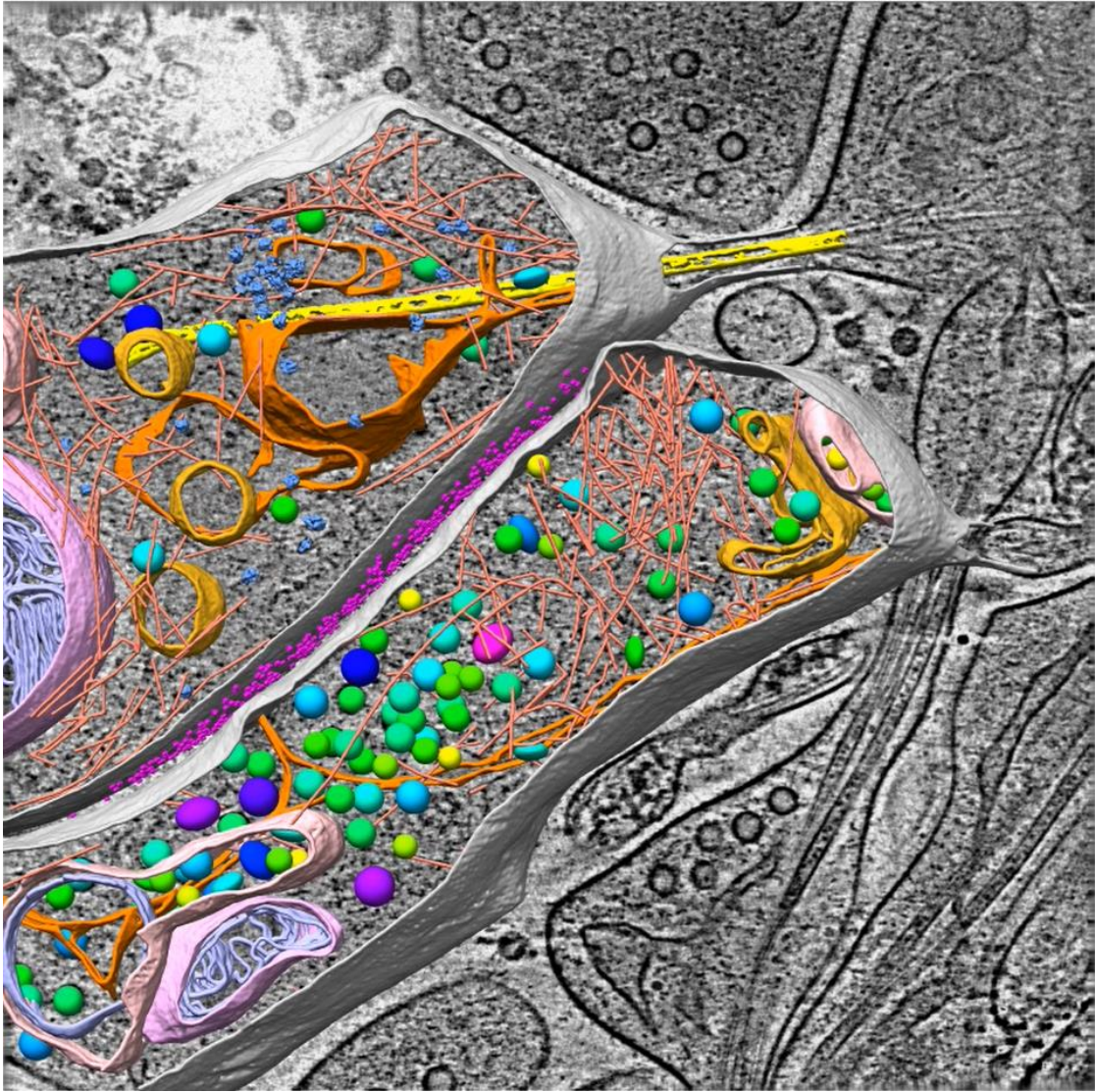
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**Supplementary Video 1 | Tilt series of an inhibitory synapse.** This video shows the tilt series of an inhibitory synapse (same data as in Fig. 1b) obtained using VPP, electron filter and counting technologies. Black dots of 15-nm diameter are gold beads used as fiducial markers for image alignment.





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**Supplementary Video 2 | Structures of an inhibitory synapse.** This video shows the tomogram of the same synapse as in Fig. 2b, displayed as z-stack and 3D surface rendering of the segmented structures. The structures were colored same as Fig. 1c and Fig. 1d.