Supplementary Materials for

Synaptic degeneration in the prefrontal cortex of a rat AD model
revealed by volume electron microscopy

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\textbf{Figure S1. Serial sections collection and image acquisition.} (A). Serial sectioning of the samples was conducted using an automated tape-collecting ultramicrotome (ATUM), and the sections were collected on tapes. (B) The tapes were segmented and attached to the silicon wafer on which the sections were imaged using a scanning electron microscope (SEM) instrument with backscattered electrons.
Figure S2. Dataset and a pipeline of 3D reconstruction and analysis. (A) An aligned 3D EM volume and cross-section in three directions of WT rat (top) and AD rat (bottom), respectively. (B) Schematic illustration of our study. Scale bar: 2 μm.
Figure S3. Reconstruction and analysis of synapses in WT and AD rats. (A). A diagram of a typical synapse, which includes a presynaptic terminal with vesicles, a synaptic cleft, and a postsynaptic unit (dendritic spine, dendritic shaft, or cell body) with PSD. (B). Ultrastructural analysis from the 2D EM images of the WT (top) and AD (bottom) rats. On the right is a high-magnification image of the red box on the left. (C). Workflow of the 3D reconstruction and analyses of PFC synapses. The red boxes
show PSDs (representing synapses), identified via a deep-learning algorithm; the sites were then replaced with the minimum enclosing rectangle to perform the 3D linkage. Each synapse was ultimately assigned a unique identifier, with the following sequence (see Materials and Methods for the details): ①. Extraction of a 2D image; ②. Synaptic site segmentation via deep learning; ③. Calculation of the minimum enclosing rectangle of the synaptic site; ④. Image filling; ⑤. 3D link of the serial 2D binary images; ⑥. 3D labels (rectangle) dot product segmentation; ⑦. 3D visualization. (D). On a 2D EM image (N =3), the number of synapses is greater in the WT than in the AD (per 100 μm²). In this and all other bar graphs, data are presented as mean ± standard deviation (Student’s t-test, **p<0.01). (E-F). Histogram and cumulative frequency of synaptic apposition surface between WT and AD rats. (11488 synapses from WT rats and 19478 synapses from AD rats were used for analysis, respectively.) (G-H). Histogram and cumulative frequency of synaptic volume between WT and AD rats. (I). Distribution and relationship between the SAS and the synaptic volume.
Figure S4. Reconstruction and analysis of presynaptic vesicle clouds in WT and AD rats. (A). Ultrastructure of synaptic junction in WT rats, including presynaptic axon terminals (cyan), vesicle clouds (yellow), mitochondria (red), and postsynaptic dendrites (green). (B). 3D ultrastructure of synaptic junction corresponds to A. (C). 3D reconstruction of presynaptic vesicle clouds in tissue volume of WT rats. (D-F). Ultrastructure in AD rats resembles A-C, respectively. (G). The density of presynaptic vesicle clouds in WT and AD rats. (H). The volume of presynaptic vesicle clouds in WT and AD rats; “×” indicates the mean. (I, J). Histogram and cumulative frequency of presynaptic vesicle clouds between WT and AD rats.
Figure S5. Reconstruction of neuronal dendrites in WT and AD rat PFC. (A). The procedure for reconstruction of neuronal dendrites. Neuronal membranes were extracted from the 3D EM images via a 3D image segmentation algorithm (3D CNN). Each neuron was then assigned a unique identifier via watershed and multicut algorithms. The dendrites of the neurons were ultimately extracted manually (see Materials and Methods for the details). (B). A total of 15 dendrites were extracted from the 3D volume images of the WT PFC. (C, D). Magnified images of 2 dendrites in the WT PFC. (E). A total of 10 dendrites were extracted from the 3D volume images of the AD PFC. (F, G). Magnified images of 2 dendrites in the AD PFC.
Figure S6. The distribution of synapses on the dendritic shafts and dendritic spines in WT and AD rats. (A, B). The synapses and dendrites from WT rats. Red indicates synapses; blue indicates dendrites. (C). The yellow box in B is magnified, showing the contact between synapses and dendritic spines. (D, E). The synapses and dendrites from AD rats. Red indicates synapses; green indicates dendrites. (F). The yellow box in E is magnified, showing the contact between synapses and dendritic shafts. (G) Schematic diagram based on the analyses of 3D images showing an increase in the number of dendritic spines and synapses formed on dendritic shafts but a decrease in the number of spine synapses in AD rats (bottom), compared with those in the WT (top). (H). Distribution of spine synapses and shaft synapses per 40 μm dendrites between WT and AD rats. The numbers next to the bars indicate the number of synapses. Data in this is presented as mean ± sd.
Table S1. The number of synapses in 2D EM images. Each number represents the number of synapses per 100 μm², and each row is randomly taken from a raw image. We randomly took three EM raw images separately from three WT rats and three AD rats.

| Number (synapse) | Volume (pixel) | Pixel resolution (nm³) | Size (sample) (μm³) | Density |
|------------------|----------------|------------------------|---------------------|---------|
| WT               | 11488          | 12858 × 12790 × 353    | 3 × 3 × 50          | 20919   | 0.549   |
| AD               | 19478          | 14416 × 14544 × 473    | 3 × 3 × 50          | 40727   | 0.478   |

Table S2. The number of 3D synapses, EM volume information, and synaptic density. Note that the sample size excludes the volume of the cell body and myelin sheath.
Materials and Methods

Animals
WT SD (Sprague Dawley) rats and App<sup>NL-G-F</sup> rats were bred in the animal facility of Tsinghua University. All rat experiments were carried out according to the guidelines of AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International). The AD rats used were App knock-in rat line harboring Swedish-Beyreuther/Iberian-Arctic mutations. The Aβ sequence was humanized by introducing mutations leading to the substitutions G676R, F681Y, and R684H (Pang et al., 2021). Rats were maintained on a standard 12 h light/12 h dark cycle. Food and water were provided ad libitum unless otherwise noted. In this study, AD rats and their WT littermates were 6-month-old male rats.

Sample fixation, sample sectioning, and wafer fabrication
The PFC sample was immersed in 4% (w/v) paraformaldehyde (PFA) and 2.5% glutaraldehyde (Sigma, G5886). After incubation in phosphate buffer (0.1M, pH7.4) with 2% OsO4 (Ted Pella, 18451) for 90 min at room temperature, the staining buffer was then replaced with phosphate buffer (0.1 M, pH 7.4) with 2.5% ferrocyanide (Sigma, 234125) for 90 min at room temperature. The samples were washed 3 times with 0.1 M phosphate buffer and incubated with filtered thio carbonylhydrazide (TCH, Sigma, 223220) for 45 min at 40 °C. Subsequently, the samples were fixed again with unbuffered 2% OsO4 for 90 min and then incubated overnight in a 1% uranyl acetate aqueous solution at 4 °C. After incubating with a lead aspartate solution (0.033 g lead nitrate (Sigma, 228621) dissolved in 5 mL of 0.03 M aspartic acid (Sigma, 11189, pH 5.0)) for 120 min at 50 °C, the sample was dehydrated with a gradient ethanol series (50%, 70%, 80%, 90%, and 100% ethanol, 10 min for each) and pure acetone. The samples were ultimately embedded with Epon 812 resin (SPI, 02660-AB).

Serial sections of the PFC sample were continuously cut with a diamond knife (Diatome, MC16425) using the automated tape-collecting ultramicrotome (ATUM, Webster et al., 2015; Baena et al., 2019) system at a cutting speed of 0.3 mm/s and collected onto a Kapton polyimide tape 8 mm wide and 100 μm thick.

The tape was segmented and attached to 4-inch silicon wafers using a double-coated carbon conductive tape (Ted Pella). The wafers were then coated with 6 nm of carbon using a high-vacuum film deposition instrument (Leica) to avoid charging.

Three-dimensional electron microscopy imaging
We selected the region of interest (ROI) in layer 4 of the PFC. To ensure the consistency of the ROI of the serial sections in the axial direction, we manually marked the central position of each section to be imaged. The ROI of each section was then imaged in the scanning electron microscope (SEM, Zeiss Gemini 300) of size [16000, 16000] at 3 nm per pixel resolution, with landing energy of 3 kV and a dwell time of 800 ns per pixel. Brightness and contrast for each ROI had been set before the central point of the ROI was manually marked. At a dwell time of 800 ns
per pixel, acquiring one ROI requires approximately 5 min.

**Image alignment**

The ATUM method was employed, and the samples were not imaged in situ, hence the nonlinear distortion of the images. Therefore, serial image alignment was indispensable in obtaining a 3D EM image stack.

A coarse-to-fine approach was adopted to align the serial images. During coarse alignment, we used a SIFT descriptor (Lowe, 2004) to extract and match the feature of each image. The random sample consensus (RANSAC) method (Fischler et al., 1981) was applied to screen out reliable matching feature point pairs to calculate the image affine deformation matrix for coarse alignment. To solve the larger miscut deformation problem during image affine transformation, we applied an alignment technique to reduce miscut deformation (Lv et al., 2020). During fine alignment, we employed a block matching-based elastic alignment method for alignment on the down-sampled 4× image (Saalfeld et al., 2012). The local correspondents in elastic alignment were checked and manually modified by the operator. Finally, we applied the deformation field obtained on the downsampled 4× image to obtain aligned 3D volume data by the original data. The image was slightly offset to produce black borders after fine alignment; thus, we intercepted the area without the black border using the entire serial image stack. Details of the WT and AD serial sections after alignment are listed in Table S3 (Figure S3).

| Data type | Section size (pixel) | Pixel resolution (nm) | Number of sections | Thickness of the sections (nm) | Sample size (µm³) |
|-----------|----------------------|-----------------------|--------------------|-------------------------------|------------------|
| WT        | 12790 × 12858        | 3                     | 353                | 50                            | 38.3 × 38.5 × 17.6 |
| AD        | 14416 × 14544        | 3                     | 473                | 50                            | 43.2 × 43.6 × 23.6 |

Table S3. Detailed description of the WT and AD 3D volume data.

**Synapse identification**

A synapse consists of a presynaptic terminal with vesicles, synaptic clefts, and postsynapses with PSD. In this study, we only identified synaptic sites without further distinguishing between presynaptic and postsynaptic targets. The features of PSD can be identified in EM images. Therefore, we trained a classifier to label two classes: background and PSD. This model applied the same encoder-decoder architecture, and the coarse-to-fine approach was adopted (Figure S7).
Firstly, to extract multi-scale features at a granular level and increase the receptive fields for each network layer, we adopted Res2Net (Gao et al., 2021) as a backbone to extract image features. Then, to integrate multi-level semantic features, we aggregated the output of Res-2, Res-3, and Res-4 through RFB-s (Liu et al., 2018). Next, we used a reverse attention module (RAM; Fan et al., 2020) to obtain object boundary accurately, which combined with multi-level features. Finally, the output of RAM was up-sampled eight times and applied a sigmoid function was to get a segmentation of the raw image.

Here, RAM can be formulated as:

$$\text{Output}^i = \text{input}^i_1 \odot (\ominus (\text{sigmoid}(\text{input}^i_2)))$$

Where $\text{input}^i_1$ denotes the output of $\text{Res}_i$, and was indicated the solid black arrow. $\text{input}^i_2$ denotes a weight map and was indicated the solid red arrow. $\ominus (\cdot)$ denotes a reverse operation subtracting the input from matrix E, in which all the elements are 1. $\odot$ is element-wise.

Ground truth labels were generated by manually labeling the PSD of the synapse for a Z slice where the PSD was visible. Thirteen slices were selected (adjacent interval 25 from the whole volume) by size $[12858, 12790]$ pixels at a resolution of $3 \times 3$ nm. Due to GPU memory constraints, each slice of labeling was cut into 169 images of size $[1024, 1024]$.

Training examples were balanced between positive examples centered on the PSD and negative examples corresponding to locations where no PSD annotations within the field of view existed. During training, we performed data augmentation, including rotations $[90 180 270]$ and reflections, along with X. Due to the sparsity of PSD annotations relative to the total number of pixels in the slices, we adopted weighted intersection over union (IoU) loss and weighted binary cross-entropy (BCE) loss to
increase the weight of difficult pixels. In addition, we adopted the strategy of calculating loss on a multi-scale to assist training. The network was trained 70 epochs by Adaptive Moment Estimation with a learning rate of 1e-4 and a batch size of 4 on a server equipped with an Intel i7 CPU, 512 GB of main memory, and an NVIDIA V100 GPU.

After predictions from the synapse detection model across the entire WT sample were generated, a unified identifier was assigned to each synapse (PSD) in the slice (generally, background: 0; synapse: 1). We measured the model on the basis of the recall rate and the precision rate of the synapse (PSD) in slices and got a 90.46% recall rate and 97.67% precision rate. We further explored that the recall rate was less than 100% because synapse at the image boundary was challenging to detect.

After identifying all synaptic sites, we discarded the synaptic sites which contained fewer than 10 pixels in each slice, which might have been falsely detected. To assign a unique identifier to each synapse in 3D, we applied a label link algorithm. More specifically, we used the minimum rectangular area (MRA) of the synapse site to represent the synapse area because of the imperfect alignment of the serial slices and a small synapse area, which can reduce errors in the link. Ultimately, the dot product was performed between the MRA and the original synapse sites (pixel value: 1).

We trained a segmentation network of presynaptic vesicle clouds based on the above model to label two classes for each pixel: background and clouds. Likewise, we used the label link algorithm to assign a unique identifier for each presynaptic vesicle cloud. Finally, 10713 and 16431 clouds of presynaptic vesicles were reconstructed in WT and AD rats, respectively.

**Dendritic reconstruction**

The reconstruction of sparse synapses is not tricky. Instead, axons and dendrites are densely packed in the tissue, dominating the volume of the cortex. Moreover, axons and dendrites in the 2D images are difficult to distinguish. In this study, we aimed to reconstruct dendrites and dendritic spines from the tissue, which was a challenge. Here, we densely reconstructed this vast majority of neurites and then extracted the dendrites and dendritic spines based on 3D morphology.

We trained a membrane segmentation network of neurites on the basis of the residual 3D-UNet architecture described in a previous study (Lee et al., 2017) to label two classes for each pixel: background and membrane. This model used the same four-down/four-up architecture described in the study mentioned above, with an initial dimensionality of 128 and a multiplicative factor of 2 for each downstage and upstage. Specifically, the input of network is a 3D image stack (serial eight images, 256 × 256 pixels). Due to the high anisotropy of serial EM images, we want to minimize the loss of information along the z-dimension. Therefore, we exclusively used 2D convolution at the highest resolution image (or feature, the convolution kernel was set 1 in the z-
In addition, Residual module # only used 2D convolution, and 3D convolution was only applied in the Residual module ##. Also, we never downsampled feature maps along the z-dimension. We also adopted skip connection in the expanding path and utilized feature addition before entering the residual module. The upsampling module used a trilinear interpolation, followed by 1×1×1 convolution to reduce the channel to keep the feature map consistent with the skip connection. Finally, the output of the network is an image stack of membrane segmentation corresponding to the input (Figure S8).

**Figure S8. The architecture of neurons segmentation network (Residual 3D U-net).** The numbers below the cubes represent the number of feature maps at each scale. The downsampling is implemented with max-pooling and upsampling with interpolation, containing two types of residual modules (Lee et al., 2017).

Ground-truth labels were generated by manually labeling the membrane of neurons, including axons, dendrites, and cell bodies. Myelin was also labeled as the membrane. However, the membranes of organelles were not labeled, which were treated as background. Five ROIs of size [2048, 2048, 30] at a resolution of 3 × 3 × 50 nm were randomly selected from the entire 3D EM volume.

To preprocess the training datasets, we first performed padding for each ROI and then cut the small patch of size [256, 256, 8] pixel by pixel for each ROI. During training, the small patches were randomly selected as the model input, and data augmentation was conducted. Data augmentation strategies consist of rotation, flip, motion blur, noise addition, small region missing, and section missing; a random technique was performed for each small patch. A batch size of 8 was used, with batch normalization applied during training, considering the enormous 3D network parameters. In addition, we used weighted BCE as the loss function in the loss calculation. The network was trained to 80000 steps at a learning rate of 10−4 with asynchronous stochastic gradient descent by using the 4 NVIDIA V100 GPU.

We generated a membrane probability map corresponding to the original WT samples.
The membrane probability map represents the probability that each pixel is a neuron membrane in the 0-1 membrane, which maps to the 8-bit image (0-255).

The membrane probability map cannot distinguish each neuron. To assign a unique identifier to each neuron, we performed a series of post-processing steps described in (Beier et al., 2017) on the membrane probability map, including a 2D watershed algorithm and hypervoxel aggregation. We primarily used a 2D watershed algorithm to generate numerous fragmented hypervoxels on the membrane probability map. Next, we constructed a graph with hypervoxels as vertices for the hypervoxels of adjacent fragments to aggregate in 3D. Ultimately the aggregation problem of hypervoxels had been transformed into a multicut problem. The edge weights of adjacent vertices were also predicted using a random forest classifier to determine whether the adjacent vertices were merged.

This study focuses on assigning a unique identifier to each neuron. Meanwhile, merge and split errors were inevitably generated from the original segmentation and the agglomeration procedure. Considering that the focus was on dendrites and dendritic spines, we extracted the dendrites of intention and proofread them.

**Three-dimensional visualization and measurements**

The software Amira (Stalling et al., 2005) was used for the 3D visualization of the synapses and dendrites. Some morphological characteristics of synapses and dendrites were conducted using the Amira software, including the 3D volume, 3D surface area, and 3D length. Further, the number of synapses was directly counted using the 3D reconstruction index, and the number of dendritic spines was calculated by skeletonization and manual counting.

**Statistical analysis**

To determine the possible differences between the AD rats and the WT rats, the synaptic density, synaptic volume, synaptic size, and the proportion of spines with synapses were evaluated using the independent two-sample Student's t-test in Microsoft Excel.

**Abbreviations**

3D = Three-dimensional; 2D = Two-dimensional; AD = Alzheimer’s disease; WT = Wild-type; PFC = Prefrontal cortex; ATUM-SEM = Automated tape-collecting ultramicrotome/scanning electron microscopy; EM = Electron microscopy; PSD = Postsynaptic density; SAS = Synaptic apposition surface; AZ = Active zone; ROI = Regions of interest; BCE = Binary cross-entropy; CNN = Convolutional neural network; MPI = Membrane probability images
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