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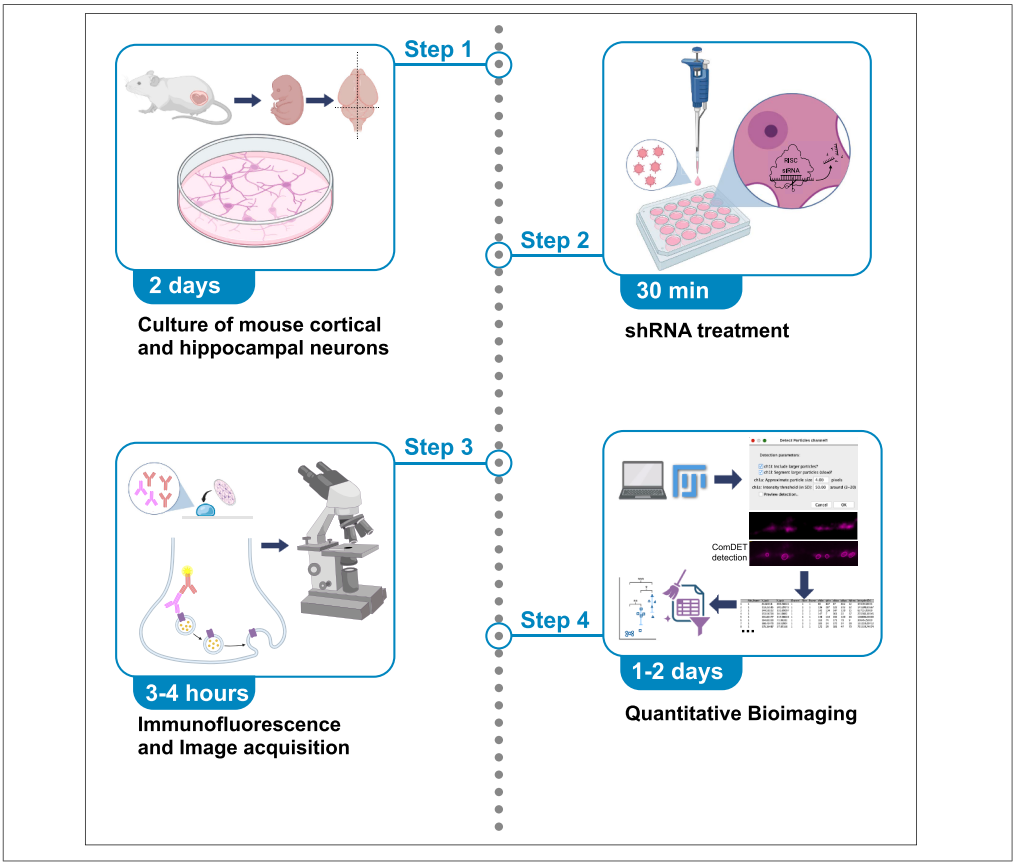
Protocol for semi-automatic quantitative bioimaging analysis of synapse loss



Synapse loss correlates with cognitive decline in neurodegenerative diseases like late-onset Alzheimer's disease. We developed a semi-automated workflow to quantify synapse loss in primary mouse neurons. The protocol includes culturing neurons on coverslips, using short hairpin RNA (shRNA)-expressing lentivirus, maintaining cultures, and labeling excitatory and inhibitory presynaptic markers by immunofluorescence. Image acquisition and analysis using Fiji/ComDet macros enable region of interest selection, neurite length measurement, puncta detection, and quantification of synapse density, size, and intensity following Bin1 knockdown.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Mariana A. Barata, Cláudia Guimas Almeida

mbarata@ed.ac.uk (M.A.B.)
claudia.almeida@nms.unl.pt (C.G.A.)

Highlights

Steps for preparing primary mouse cortical and hippocampal neurons

Instructions for Bin1 shRNA lentiviral transduction of cultured neurons

Guidance on immunostaining for inhibitory and excitatory synaptic markers

Steps for Fiji- and ComDet-based quantification of synapse density and colocalization

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Mariana A. Barata[1,2,3,*] and Cláudia Guimas Almeida[1,4,*]

[1]iNOVA4Health, NOVA Medical School, Universidade NOVA de Lisboa, 1169-056 Lisboa, Portugal

[2]Present address: Institute for Neuroscience and Cardiovascular Research, Hugh, Robson Building, George Square, University of Edinburgh, Edinburgh, EH8 9XD, Scotland, UK

[3]Technical contact

[4]Lead contact

*Correspondence: mbarata@ed.ac.uk (M.A.B.), claudia.almeida@nms.unl.pt (C.G.A.)
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SUMMARY

Synapse loss correlates with cognitive decline in neurodegenerative diseases like late-onset Alzheimer's disease. We developed a semi-automated workflow to quantify synapse loss in primary mouse neurons. The protocol includes culturing neurons on coverslips, using short hairpin RNA (shRNA)-expressing lentivirus, maintaining cultures, and labeling excitatory and inhibitory presynaptic markers by immunofluorescence. Image acquisition and analysis using Fiji/ComDet macros enable region of interest selection, neurite length measurement, puncta detection, and quantification of synapse density, size, and intensity following Bin1 knockdown.

For complete details on the use and execution of this protocol, please refer to Barata et al.[1]

BEFORE YOU BEGIN

Identifying the underlying mechanisms of synapse loss in Alzheimer's disease (AD) is a critical step toward developing effective therapeutic strategies. In early-onset familial AD, monogenic mutations deregulate beta-amyloid (Aβ) production and potentiate Aβ aggregation, which affects both excitatory[2–10] and inhibitory synapses.[2,11,12] Dysfunction of excitatory synapses reduces long-term potentiation and contributes to memory deficits in AD.[13–15] Conversely, defects in inhibitory synapses drive neuronal hyperexcitability, leading to hyperactivity and seizures in AD.[2,14,16–19]

This protocol describes specific steps to assess the contribution of the endocytic regulator bridging integrator 1 (Bin1), a major risk factor for late-onset AD (LOAD),[20,21] to synapse loss in primary mouse neurons. After preparing mouse neuronal cultures from embryonic BALB/c mice, Bin1 is knocked down using a lentiviral shRNA. Next, we use Fiji software (https://fiji.sc/), an open-source platform for scientific image analysis, together with the ComDet plugin and custom macros to enable rapid quantitative analysis of synapses. We employed a macro to select and crop regions of interest (ROI) for neuronal process segments and measure their lengths. On the ROIs, we use a custom macro with the comDET plugin to quantify synapse number, size, and intensity. Synapse density is then calculated by dividing the number of synaptic puncta per 50 μm of axonal length. We use this protocol to quantify synapse loss with neuronal aging, other risk genes, and in NRF2KO mice[1,22]

Innovation

Our methodology involves using the open-source ImageJ plugin ComDET to segment synaptic puncta and measure object-based colocalization, size, intensity, and number. ComDet enables

visualization and optimization of puncta detection. Additionally, we employ an in-house FIJI macro that streamLines and automates image preprocessing, thresholding, ROI handling, and batch export of ComDet outputs. This semi-automatic, user-friendly protocol requires no programming, enabling users with minimal computational experience to perform standardized, reproducible synapse quantification. This makes our protocol accessible to laboratories without bioinformatics support.

Institutional permissions

All animal procedures should be performed in accordance with EU recommendations and approved by local ethical committees. The Ethical Committee and the Animal Welfare Body (ORBEA) of the NMS approved our work – Universidade Nova de Lisboa (162/2021/CEFCM).

Prepare shRNA-expressing lentivirus

Timing: 1–2 weeks

This step describes the production and concentration of shRNA-expressing lentivirus for neuronal transduction.

1. Co-transfect in STAR-RDpro cells shRNA vectors against Bin1 or non-targeting with psPAX2 and pMD2.G, along with helper plasmids.
2. Collect viral supernatants by centrifugation.
3. Concentrate lentiviral particles with the Lenti-X concentrator according to the manufacturer's protocol.
4. Resuspend pellet viral particles in neuronal media and store at −80°C until use.
5. Titrate each lentiviral batch to determine the optimal transduction volume that balances GFP expression with minimal Bin1 immunofluorescence.

Prepare coated coverslips

Timing: 1 week

To prepare acid-washed sterile poly-D-lysine (PDL) coated 13-mm glass coverslips for neuronal culture (Figure 1A).

6. Treat glass coverslips for 1 h with 40% ethanol/60% HCl to improve cell adhesion.
7. Wash coverslips 3 times with ddH2O, then a final wash with 96% ethanol to facilitate drying.

Note: Spread coverslips apart to prevent sticking.

8. Air-dry and sterilize, covered with aluminum foil, in a dry-heat oven.
9. 24 h before dissection, coat the coverslips with poly-D-lysine.
   a. In a laminar-flow cabinet, place treated coverslips in a 24-well plate.
   b. Incubate 16 h with 0.1 mg/mL PDL (500 μL per well) at 37°C in 5% CO_2.
10. Wash 3 times with sterile ddH_2O, then allow to dry in the hood or for 16 h at 37°C in a 5% CO_2 incubator.

Note: Coverslips should be dry before proceeding.

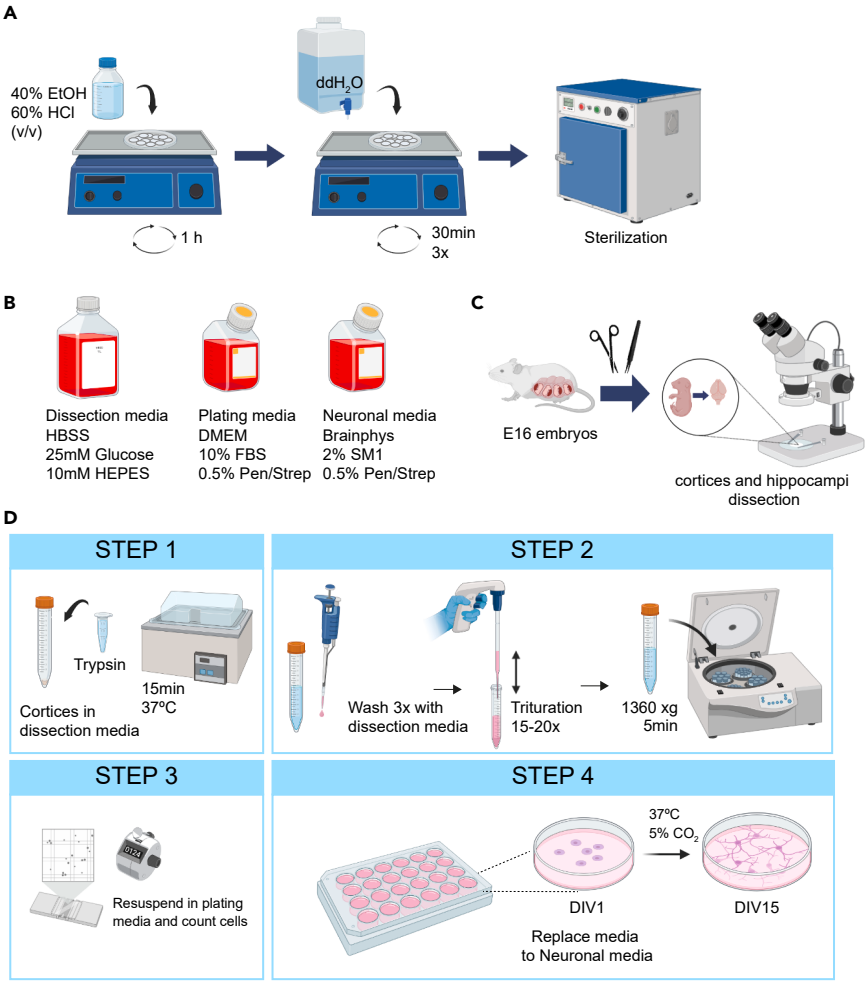

Figure 1. Preparation of cultures of mouse primary cortical and hippocampal neurons

(A) Preparation of 13-mm glass coverslips for neuronal culture by acid wash.

(B) Preparation of dissection, plating, and neuronal media.

(C) Dissection of cortices and hippocampi from wild-type BALB/c mice (embryonic day 16, either sex).

(D) Dissociation of dissected cortices and plating primary mouse neurons.

Step 1: trypsinization of brain tissue.

Step 2: washing, trituration, and pellet dissociation of tissue.

Step 3: Count neuronal cells.

Step 4: Plate neurons in a 24-well plate with a PDL-coated coverslip. At DIV (days *in vitro*) 1, replace plating media with neuronal media. Maintain neurons at 37°C in 5% CO_2 until experimental day (DIV 14). Created with BioRender.com.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Guinea Pig polyclonal anti-vGLUT1 1:200 (IF), 1:500 (IHC)	Synaptic Systems	Cat#135 304; RRID:AB_887878
Rabbit polyclonal anti-vGAT 1:500 (IF), 1:200 (IHC)]	Merck Millipore	Cat#AB5062P; RRID:AB_2301998
Chicken polyclonal anti-MAP2 1:500 (IF)	Abcam	Cat#ab5392; RRID:AB_2138153
Mouse monoclonal anti-Bin1 1:100 (IF), 1:1000 (WB), 1:80 (IHC)	Merck Millipore	Cat#05-449; RRID:AB_309738

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Fetal Bovine Serum, qualified, heat-inactivated, United States	Thermo Fisher Scientific	Cat#16140071
Hydrochloric Acid 37%, for analysis, ACS, ISO	PanReac AppliChem	Cat#131020
99.9% (v/v) pure Ethyl Alcohol	Manuel Viera & Cª (Irmão) Sucrs, Lda	N/A
Trypsin (2.5%), no phenol red	Thermo Fisher Scientific	Cat#15090046
Poly-D-lysine hydrobromide	Sigma-Aldrich	Cat#P1149
Hank's Balanced Salt Solution (HBSS), no calcium, no magnesium	Thermo Fisher Scientific	Cat#14170112
D(+) Glucose Anhydrous	NZYtech	Cat#MB16801
Phosphate-Buffered Saline (PBS), pH 7.4	Thermo Fisher Scientific	Cat#10010031
HEPES (1M)	Thermo Fisher Scientific	Cat#15630080
BrainPhys™ Neuronal Medium and SM1 Kit	STEMCELL Technologies	Cat#05792
Penicillin-streptomycin (10,000 U/mL)	Thermo Fisher Scientific	Cat#15140122
Lenti-X™ concentrator	Takara Bio	Cat#631231
Paraformaldehyde 95%, powder	Sigma-Aldrich	Cat#158127
Sucrose	NZYtech	Cat#MB18601
Triton™ X-100	Sigma-Aldrich	Cat#T9284
Saponin	Sigma-Aldrich	Cat#47036
Fluoromount-G™ Mounting Medium	Thermo Fisher Scientific	Cat#00-4958-02
4′,6-diamidino-2-phenylindole (DAPI)	Sigma-Aldrich	Cat#D9542
Experimental models: Organisms/strains		
E16 pregnant females' mice (BALB/c)	Charles River	BALB/cByJ; RRID:IMSR_JAX:001026
STAR-RDpro	ECACC	Cat#04072117, RRID:CVCL_AR78
Recombinant DNA		
shBin1-1 (Mission® shRNA)	Sigma-Aldrich	TRCN0000380439
shBin1-2 (Mission® shRNA)	Sigma-Aldrich	TRCN0000088191
Mission® pLKO.1-PURO-CMV-TurboGFP™ Positive Control Plasmid DNA)	Sigma-Aldrich	Cat#SHC003
pGFP-C-shLenti	OriGene Technologies	Cat#NC1766994
psPAX2	Didier Trono lab(EPFL)	Cat#12260; RRID:Addgene_12260
pMD2.G	Didier Trono lab(EPFL)	Cat#12259; RRID:Addgene_12259
Software and algorithms		
Fiji (version 1.53)	NIH	https://fiji.sc/ ; RRID:SCR_002285
ComDet v.0.5.5	*Katrukha*[23]	https://github.com/ekatrukha/ComDet
GraphPad (version 8.4.0)	Dotmatics	www.graphpad.com; RRID:SCR_002798
RDB Merge (Excel Add-In)	Ron de Bruin	RDBMerge, Excel Merge Add-in for Excel for Windows
Other		
Round Cover glasses, ⌀ 13 mm	Marienfeld	Cat#0111530
Cell culture plate, 24 well, surface: Cell+, flat base	Sarstedt	Cat#83.3922.300
Petri dish, 92 × 16 mm	Sarstedt	Cat#82.1473.001

MATERIALS AND EQUIPMENT

Dissection media

Reagent	Final concentration	Amount
HBSS	N/A	49 mL
Glucose	25 mM	0.225 g
HEPES	10 mM	500 µL
total	–	**50 mL**

Prepare fresh before each experiment, store at 4°C for up to 1 week.

Plating media		
Reagent	Final concentration	Amount
Dulbecco's medium Eagle medium (DMEM)	N/A	45 mL
Fetal bovine serum (FBS)	10%	5 mL
Penicillin-streptomycin (Pen/Strep)	0.5%	250 µL
total	–	**50 mL**
Prepare fresh before each experiment, store at 4°C for up to 1 week.		

Neuronal media		
Reagent	Final concentration	Amount
Brainphys Neuronal Medium	N/A	49mL
SM1	2%	1mL
Penicillin-streptomycin Pen/Strep)	0.5%	250 µL
total	–	**50 mL**
Prepare fresh before each experiment, store at 4°C for up to 2 weeks. The remaining solution can be used for transductions.		

STEP-BY-STEP METHOD DETAILS

Prepare cultures of mouse primary cortical and hippocampal neurons

Timing: 2 days

This step describes the dissection of cortices and hippocampi from embryonic mice, followed by tissue dissociation and plating of primary cortical and hippocampal neurons.

1. Dissect cortices and hippocampi from wild-type BALB/c mice (embryonic day 16, either sex) (E16) (Figure 1C).
   a. Place plating and neuronal media at 20°C and dissection media on ice (Figure 1B).
   b. Thaw trypsin (2,5% no phenol red) at 4°C.
   c. Euthanize the dam in accordance with the guidelines approved by the researcher's institution.
   d. Spray the abdominal skin with ethanol, hold the skin with forceps, perform an incision with scissors, and remove the uterus.
   e. Free individual fetuses in ice-cold dissection media on a petri dish on ice and quickly proceed with decapitation.
   f. Dissect brains individually under a stereomicroscope in a petri dish with ice-cold dissection media.
   g. Release the brain using two forceps; one holds the brain by the eyes, and the other pulls the skin at the confluence of sinuses (COS).

   Note: With one forceps secured at the COS, the other can be used to gently pry the overlying membranes outward in a semicircular motion and expose the brain.

   h. Separate cortices from the cerebellum and brain stem, split hemispheres, and remove meninges.
   i. Place dissected cortices in one 15 mL conical tube containing 1.8 mL ice-cold dissection media (4 hemispheres per tube).
2. Dissociate brain tissue and plate neurons (Figure 1D):
   a. Allow for trypsinization by adding 200 µL of trypsin to each tube and placing it in a water bath at 37°C for 15min.
   b. Allow the tissue to settle, then carefully remove the trypsin.
   c. Carefully wash the tissue 3 times, adding 1.5 mL of dissection medium each time.

d. Remove the dissection medium. Add 1.5 mL of plating medium and gently triturate the tissue with a glass Pasteur pipette to dissociate cells.

Note: Avoid generating bubbles. Triturate gently (pipetting up and down, up to 20 times) by touching the bottom of the tube.

e. Pool the dissociated tissue into a single 15 mL tube and centrifuge at 1360 × *g* for 5 min at RT.
f. Resuspend the pellet in 5 mL of plating medium.
g. Perform a 1/20 dilution by taking 20 μL of cell suspension into 180 μL of plating media, then taking 10 μL into 10 μL of trypan blue.
h. Count cells on a hemocytometer.
i. Plate 5×10^4 neurons in 500 μl per well of a 24-well plate with a PDL-coated coverslip.

Note: Mix cells before plating by gently flicking or inverting rather than by pipetting up and down.

j. Maintain neurons at 37°C in 5% CO_2.

3. The next day, replace the plating medium with 500 μL of pre-warmed neuronal medium for survival and differentiation (Figures 1D and 2).

Knockdown with lentivirus

Timing: 30 min

This step introduces shRNA constructs to knockdown Bin1 expression in primary neurons. Lentiviral delivery ensures efficient and stable transduction, and keeping the conditioned media supports neuronal survival during recovery from infection.

4. At 8 days in vitro (DIV), assess the quality of the plated neurons by microscopy. If neurons are healthy, with developed and unbroken processes, transduce primary neurons with a lentiviral vector expressing shRNA targeting BIN1 or a non-targeting shRNA (control).

Note: We use two Bin1-specific shRNA sequences.

a. Collect and reserve 300 μL of conditioned medium per well.
b. Apply lentiviral particles dropwise to each well, then incubate for 5 min at 37°C in 5% CO_2.
c. Remove the infection medium and replace it with previously collected conditioned media.
d. Complete with freshly prepared neuronal media for a final volume of 500 μL per well.
e. Maintain neurons at 37°C in 5% CO_2 until 15 DIV for downstream experiments.

Immunofluorescence

Timing: 3–4 h

This step labels synaptic markers in cultured primary neurons to visualize synapses and measure puncta density, size, and intensity. Proper fixation, permeabilization, and blocking are essential to preserve neuronal morphology and minimize non-specific antibody binding. Optimizing primary antibody concentration is crucial for providing a strong, specific fluorescent signal with minimal background. All steps are performed at 20°C.

5. Fix cultured primary neurons with 4% paraformaldehyde and 4% sucrose in PBS for 20 min in a hood.

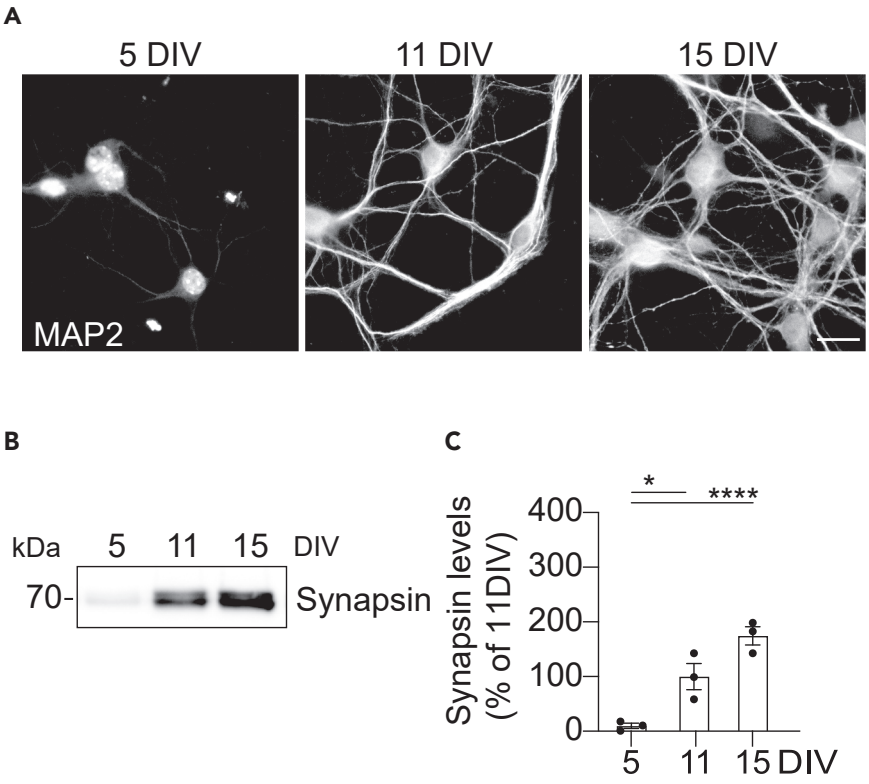

Figure 2. Differentiation of primary mouse neurons at DIV 5, 11, and 15

(A) Staining for MAP2 shows progressive neuronal maturation over time in culture with well-developed axonal and dendritic arbors by DIV 15.

MAP2-labeled neurites appear continuous and unfragmented, and progressively more complex over time in culture. Scale bar, 20 μm.

(B) Synaptic maturation with increased synapsin I levels over time in culture.

(C) Synapsin I levels normalized to 11 DIV. *p = 0.0210 synapsin11DIV vs. synapsin5DIV, ***p = 0.0005 synapsin15DIV vs. synapsin5DIV; two-way ANOVA, mean ± SEM. Figure reprinted and adapted with permission.[1]

Note: Add or remove fixative carefully using a transfer pipette against the side of the well to avoid disturbing the neurons.

6. Wash each well twice with PBS. Discard paraformaldehyde waste in a dedicated container.

Pause point: The plates can be stored at 4°C for up to 2 weeks.

7. Permeabilize by incubation with 0.3% Triton X-100 in PBS for 5 min.
8. Block in 0.1% saponin, 2% FBS, 1% bovine serum albumin (BSA) in PBS for 1 h to reduce non-specific binding.

Note: Continue to add solutions using a transfer pipette; however, vacuum aspiration with a pipette tip may be used, always against the side of the well to avoid disturbing the neurons.

9. Incubate with primary antibodies against synaptic markers, VGAT for inhibitory synapses and VGLUT1 for excitatory ones, for example (Figure 3A).
   a. Prepare diluted primary antibodies in the blocking solution.
   b. Prepare a humid chamber to prevent samples from drying out during antibody incubation.

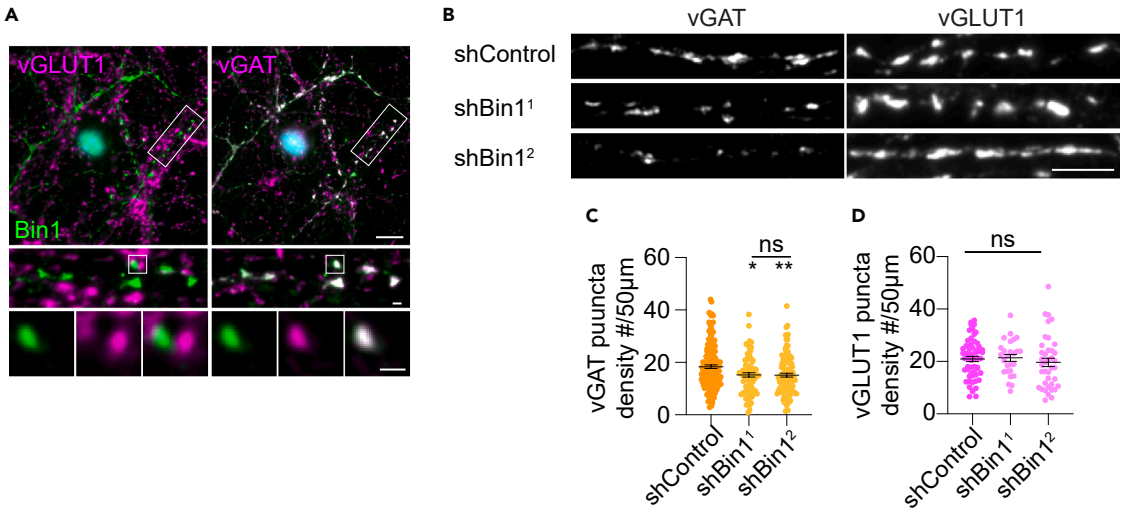

Figure 3. Expected outcomes for immunofluorescence and quantification of synaptic puncta

(A) Immunofluorescence of Bin1 (green) and vGLUT1 or vGAT (magenta) of mature (DIV 15) primary mouse neurons. Neurons exhibit a dense network of vGLUT1-positive excitatory puncta and vGAT-positive inhibitory puncta. Immunostaining produces clearly resolved synaptic puncta. Scale bars, 10 µm, 1 µm (insets).

(B–D) Presynaptic density of vGAT and vGLUT1. Scale bar, 5 µm (A).

Density of vGAT. n = 3–5; N = 28–168 axons; Mann-Whitney test vs. shControl (C), vGLUT1. *p = 0.0399 shBin11, **p = 0.0079 shBin12, ns, non-significant, mean ± SEM (D). Figure reprinted and adapted with permission[1].

Note: A humid chamber can be made with wet paper in a sealed box. Place parafilm on top of the wet paper. Place a 25 µL drop of primary antibody solution on a parafilm-coated plate for each coverslip; 25 µL is sufficient for a 13-mm glass coverslip.

c. Retrieve the coverslips using forceps, and place them onto drops of primary antibody solution, and incubate for 1 h at 20°C.

10. Wash by transferring coverslips to wells of a 24-well plate filled with 1× PBS. Wash two more times with 1× PBS.
11. Incubate with corresponding secondary antibodies.
    a. Dilute secondary antibodies (1:250) and DAPI (1:100) to label nuclei in the blocking solution.
    b. Place a 25 µL drop of secondary antibody solution on a parafilm-coated plate for each coverslip.
    c. Retrieve coverslips using forceps, place them onto the surface, and incubate for 1 h at 20°C.

Note: Minimize light exposure during and after the secondary antibody step to prevent photobleaching.

    d. Wash cells twice with sterile 1× PBS.
12. Mount coverslips using Fluoromount-G in glass slides.
13. Air-dry at least 16 h. Store at 4°C for long-term storage.

Note: Imaging before drying is possible by sealing the edges of the coverslips with nail polish to prevent movement.

Image acquisition

Timing: Approximately 10 min per coverslip

This step images fluorescently labeled synaptic markers in neurons for quantitative analysis. Keeping imaging parameters constant allows comparison between conditions.

14. Perform epifluorescence microscopy on a wide-field upright microscope, such as Axio Imager Z2 (Zeiss, Oberkochen, Germany), equipped with a 63× NA 1.4 oil-immersion objective and an AxioCam MRm charged-coupled device (CCD) camera (Zeiss).
    a. Find GFP-expressing neurons.
    b. Acquire single images- If cells are 3-dimensional, acquire a z-stack image (optimal interval).
    c. Image all samples in parallel and using identical acquisition parameters.
    d. Save 16-bit CZI (or TIFF) file format.

⚠ CRITICAL: Adjust exposure time for synaptic puncta to avoid saturation of the fluorescent signal to ensure accurate downstream quantification of puncta size and intensity.

Install Fiji, plugins, and macros

Timing: 1–2 days

This step allows the installation of the image analysis software and the required plugins to quantify the number, size, and intensity of synaptic puncta in neuronal processes (Figure 4; see also Methods videos S1 and S2). Use the macro to enable batch processing, allowing consistent ROI-based cropping of large datasets with user control over region selection. Access macro code in GitHub depository: https://github.com/neuroagingandAD/Synapsepunctaanalysis.git.

15. Install FIJI on your computer and the ComDet plugin following the website instructions.
16. Open Step 1 macro (Step1_macro.ijm), written in ImageJ Macro language, to create regions of interest for synaptic puncta analysis (Figure 4, Methods video S1).
    a. Create a folder per experiment (e.g., Experiment01_shBin1_VGAT_VGLUT)
    b. In this experiment folder, save all images for one experiment (e.g., E1_input) in a "input" labeled folder.
    c. Create a "ROIs Folder" to save the output images.
17. Run the Step1_macro.
    a. Import raw multichannel microscopy images (input) via Bio-Formats to preserve metadata and channels.

⚠ CRITICAL: To open.czi images, use the Bio-Formats Importer.

    b. Adjust the image to make it easier to inspect in the Channels Tool and Brightness/Contrast dialog (Figure 4, step 2).

⚠ CRITICAL: If z-stacks were acquired, duplicate the slice from the Z-stack with the best focal plane.

    c. Subtract background to enhance puncta detection. Adjust the rolling ball radius (e.g., 50) parameter as needed (Figure 4, step 2.2).
    d. Manually select ROIs on synapse-bearing neuronal segments using the polygon selection tool (Figure 4, step 3).
    e. Save ROIs automatically as unaltered TIFFs (ROIs Folder), with filenames linked to the originals to ensure traceability, for use as masks in analysis.
    f. Export the Feret measurements for each ROI for puncta density analysis (Figure 4, step 4).

Note: Use print("Processing file.") to save the image name and ComDet parameters to a.txt log file.

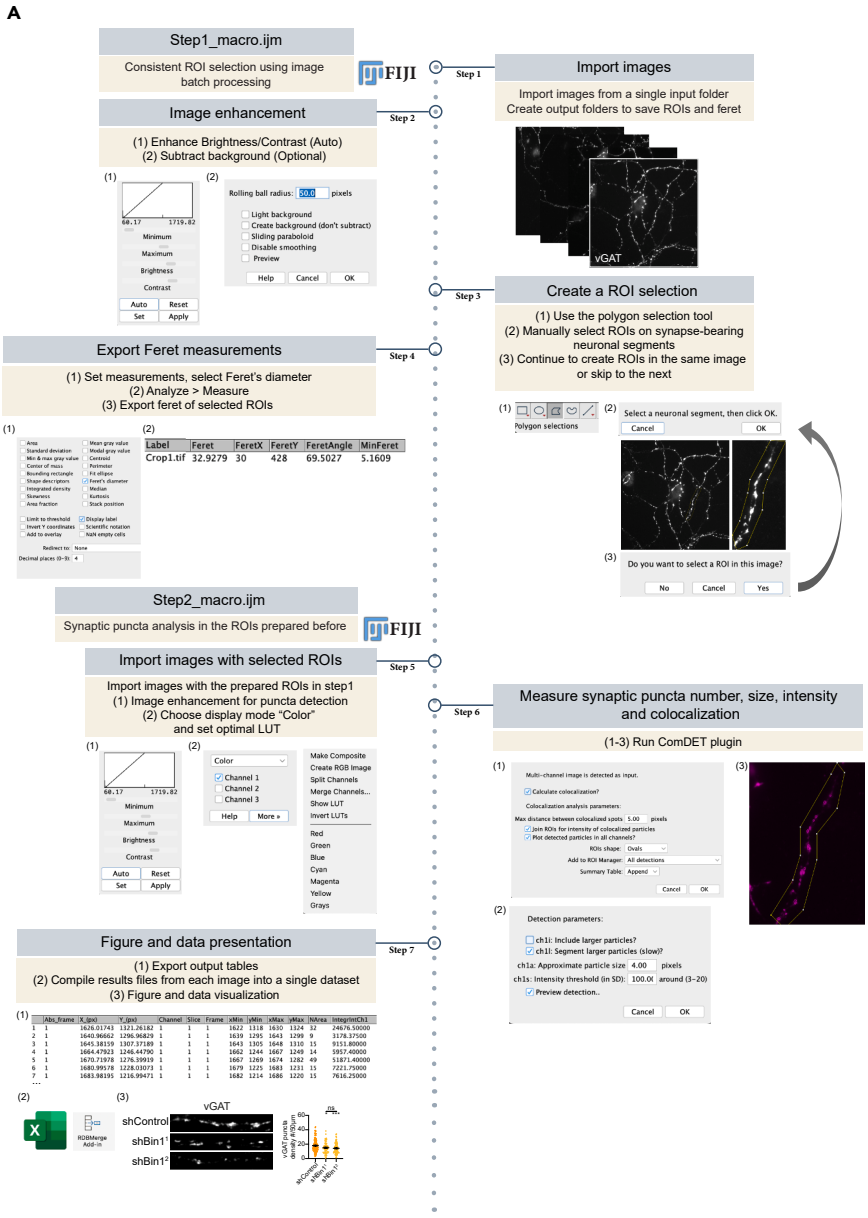

Figure 4. Macro-based workflow for batch image processing in FIJI

The pipeline is divided into two macros (step1_macro.ijm and step2_macro.ijm). Macro 1 imports raw images, performs automated brightness/contrast adjustment and background subtraction, enables manual ROI selection on synapse-bearing neuronal segments, and extracts Feret measurements.

The selected ROIs are then reloaded in Step 2 for synaptic puncta detection and quantification with the ComDet plugin, which measures puncta number, size, intensity, and colocalization.

Output tables are compiled across images for downstream analysis and visualization.

18. Run the Step2_macro (Step2_macro.ijm) for synaptic puncta analysis in the ROIs prepared before (Figure 4, Methods video S2).
    a. Batch-Import multichannel images with previous neurite selections into Fiji via Bio-Formats.

Note: Display in composite display mode in a chosen LUT color to optimize visualization of synaptic puncta (E.g., green and magenta appear white when colocalized (Figure 4, step 5).

b. Run particle/puncta detection (ComDet "Detect Particles") and perform detection on each channel.

Note: The plugin does not switch channels in the composite display mode.

c. Set colocalization distance and segmentation parameters (intensity threshold and particle size) consistently across images, making minor adjustments if necessary (Figure 4, step 6.2).

Note: On ComDet dialog: Tick "Calculate colocalization?" and set "Max distance between colocalized spots = 5 pixels (Figure 4, step 6.1).

⚠ CRITICAL: Tick "Append" to combine colocalization analysis across images.

d. Save automatically ComDet output tables per image and a global Summary/Log output (when generated) at the end of the batch run.

EXPECTED OUTCOMES

We established this protocol to measure synapse density in mature mouse primary neurons and to assess synapse loss associated with the LOAD risk gene Bin1. Using this protocol, we expect to produce cortical and hippocampal neurons with a dense network of vGLUT1-positive excitatory puncta (80%) and vGAT-positive inhibitory puncta (20% of culture). Neurons should attach evenly across poly-D-lysine-coated coverlips, show appropriate levels of differentiation at DIV8, and fully mature at DIV15 with well-developed axonal and dendritic arbors (Figure 2).

Lentiviral transduction at DIV8 should achieve robust shRNA expression, with minimal cytotoxicity and at least a 50% reduction in Bin1 expression, as verified by immunostaining or Western blotting.

By DIV15, immunostained neurons should exhibit clearly resolved excitatory (e.g., vGLUT1-positive) and inhibitory (e.g., vGAT-positive) synaptic puncta with negligible bleed-through between channels (Figure 3A). MAP2 can be used to assess neuronal health, as staining should be continuous rather than fragmented (Figure 2). A nuclear staining (DAPI) highlights neuronal cell bodies.

Proper image acquisition should yield well-focused z-stacks that capture all synaptic structures without overexposing fluorescence. Quantitative bioimaging using FIJI/ImageJ and the ComDet plugin should generate reproducible measurements of synapse density, size, and intensity. Researchers following this protocol should be able to produce data suitable for statistical analysis of synaptic alterations, including comparative studies between gene knockdown and control conditions (Figures 3B–3D). BIN1 knockdown selectively reduced inhibitory synapse density while sparing excitatory synapses, consistent with a specific vulnerability of inhibitory terminals (Figures 3B–3D).[1] Users should be able to reproduce similar effect directions and magnitudes across independent experiments when culture quality and imaging parameters are well controlled. Figures generated from these data typically include representative immunofluorescence images, segmented puncta overlays, and summary graphs showing synaptic density, size, and colocalization metrics.

QUANTIFICATION AND STATISTICAL ANALYSIS

Compile results files from each image into a single dataset using the Excel Merge Add-In, RDB Merge, following the developer's instructions (Figure 4, step 7).

⚠ CRITICAL: Tick on "Add file name". Clean up the dataset tables by removing duplicate headers, blank rows, and data for channels not analyzed. Convert ROI areas in pixels to μm^2 by using the image calibration (FIJI > Image > Properties). Use a dynamic table to

summarize results per image/neurite, then calculate puncta intensity relative to the control condition and puncta density expressed as the number of puncta per 50 μm using the ROI Feret's diameter extracted.

⚠ CRITICAL: Ensure that intensity measurements correspond to the correct channel; e.g., puncta intensity for channel 1 should be obtained from the "integrIntCh1" column.

Colocalization analysis is available in the generated "Summary" file. Additionally, it can be derived from the "Results" file by filtering the ColocCh1 or ColocCh2 columns for values of "1" (colocalized) or "0" (not colocalized). Once intensity, density, and colocalization data are obtained, perform statistical comparisons between experimental groups (e.g., Bin1 knockdown vs control) using appropriate tests based on the data distribution. Present data as mean ± standard deviation or standard error with significance indicated where relevant. Use at least 3 independent cultures with 10–15 images per condition.

LIMITATIONS

While this protocol allows quantitative analysis of synapse density, size, intensity, and colocalization in primary mouse neurons, several limitations should be considered. First, the quality of neuronal cultures can vary depending on embryonic dissection skill, cell density, and plating conditions; low cell survival or poor neurite outgrowth can reduce the reliability of synaptic measurements. Second, lentiviral transduction efficiency may also vary between preparations, affecting the consistency of gene knockdown and downstream results. Third, during immunofluorescence, incomplete fixation, uneven antibody penetration, or non-specific binding can introduce variability in signal intensity and puncta detection. Image acquisition relies on proper z-stacking and focus selection; errors in focal plane selection, photobleaching, or oversaturation can compromise quantitative analysis. Fourth, this protocol is optimized for puncta detection along axonal and dendritic processes, where individual puncta can be resolved. Analysis of neuronal somata is more challenging because diffuse somatic staining can reduce the accuracy of particle segmentation; somatic analysis should therefore be optimized and validated separately. Finally, while the ComDet plugin and FIJI macros facilitate automated detection, parameter optimization is critical, and small changes in thresholding or ROI selection can affect the reproducibility of puncta counts and colocalization measurements.

This protocol relies on dissociated primary mouse neurons, which cannot fully recapitulate network architecture and neuromodulatory inputs present in vivo. As a result, culture conditions and time in vitro can influence synapse density and maturation. In addition, our readouts are based on fixed-cell immunostaining and object-based colocalization, which capture structural, but not functional, aspects of synaptic dynamics and vesicle recycling. Thresholding and segmentation parameters in ComDet must be kept constant across conditions; small changes can affect absolute puncta counts, although relative differences between conditions are robust when imaging and analysis parameters are standardized. Complementary electrophysiological recordings or live-cell imaging can be used to validate functional consequences of BIN1 manipulation at inhibitory synapses.

TROUBLESHOOTING

Problem 1

Poor neuron survival, with primary neurons showing high cell death or failing to attach and grow properly (Step 6–13).

Potential solution

Check coverslip preparation and coating. Ensure that coverslips are fully submerged in the poly-D-lysine solution during coating, and allow them to dry completely before plating in step 12i. Use freshly prepared culture media and handle cells gently during dissociation and plating. Avoid excessive mechanical trituration in step 12d. Prefer plating cells using 10/5mL pipettes rather than small

micropipette tips to avoid mechanical stress. Maintain stable temperature and CO_2 conditions throughout culture.

Problem 2

Lentiviral shRNA transduction does not yield a clear GFP signal for identifying transduced neurons (Step 14).

Potential solution

Confirm knockdown efficiency by western blot or qPCR. If necessary, adjust fluorescence acquisition settings in step 24 to enhance the GFP signal, or optimize viral titer and infection duration in step 14 to improve neuron identification for imaging analysis. Ensure that neurons are healthy and confluent at the time of infection (DIV8).

Problem 3

Macro script does not execute as expected or produces errors (Step 25–28).

Potential solution

Inspect the macro code for formatting issues, such as missing brackets. Use Plugins > Macro > Record in FIJI to record manual steps and generate the corresponding macro commands, and compare them to the macro script. Consult the Image.sc forum for specific troubleshooting tips and examples.

Problem 4

The immunofluorescence signal is weak or non-specific, or exhibits a high background, resulting in poor puncta detection (Step 15–23).

Potential solution

Optimize fixation, permeabilization, and blocking conditions in steps 15, 17, and 18. Adjust the primary and secondary antibody dilutions or extend the incubation from 1 h to 16 h at 4°C in steps 19 and 21. Ensure consistent washing with sufficient volumes and time. Include negative controls (no primary antibody) in step 19 to distinguish true signal from non-specific binding.

Problem 5

How the image analysis pipeline can be adapted for use with different samples, e.g., brain sections.

Potential solution

For brain sections, acquire confocal images with consistent settings to minimize out-of-focus signal, improve resolution, and reduce background. Define smaller regions of interest (e.g., by selecting a square area) to focus analysis on well-resolved regions and reduce processing time. Additionally, optimal background subtraction in step 27c helps improve the signal-to-noise ratio and improve puncta detection. Once optimized, the same imaging settings, ROI selection criteria, background subtraction, thresholding, particle-size parameters, and colocalization distance should be kept constant across all experimental conditions.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Cláudia Guimas Almeida (claudia.almeida@nms.unl.pt).

Technical contact

Questions about the technical specifics of performing the protocol should be directed to the technical contact, Mariana A. Barata (mbarata@ed.ac.uk).

Materials availability

This study did not generate new, unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- All original code has been deposited at GitHub depository and is publicly available at http://github.com/neuroagingandAD/Synapsepunctaanalysis.git as of the date of publication.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Methodology, M.A.B.; writing – original draft, M.A.B.; writing, M.A.B. and C.G.A.; visualization, M.A.B.; funding acquisition, C.G.A.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2026.104675.

REFERENCES

1. Barata, M.A., Perdigão, C., Ramalho, J., Gomes, E.R., and Guimas Almeida, C. (2025). Alzheimer's genetic risk factor Bin1 controls synapse vesicle exo-endocytosis in inhibitory synapses. Cell Rep. *44*, 116005. https://doi.org/10.1016/j.celrep.2025.116005.

2. Palop, J.J., and Mucke, L. (2010). Amyloid-β–induced neuronal dysfunction in Alzheimer's disease: from synapses toward neural networks. Nat. Neurosci. *13*, 812–818. https://doi.org/10.1038/nn.2583.

3. Perdigão, C., Barata, M.A., Araújo, M.N., Mirfakhar, F.S., Castanheira, J., and Guimas Almeida, C. (2020). Intracellular Trafficking Mechanisms of Synaptic Dysfunction in Alzheimer's Disease. Front. Cell. Neurosci. *14*, 72. https://doi.org/10.3389/fncel.2020.00072.

4. Almeida, C.G., Tampellini, D., Takahashi, R.H., Greengard, P., Lin, M.T., Snyder, E.M., and Gouras, G.K. (2005). Beta-amyloid accumulation in APP mutant neurons reduces PSD-95 and GluR1 in synapses. Neurobiol. Dis. *20*, 187–198. https://doi.org/10.1016/j.nbd.2005.02.008.

5. Ting, J.T., Kelley, B.G., Lambert, T.J., Cook, D.G., and Sullivan, J.M. (2007). Amyloid precursor protein overexpression depresses excitatory transmission through both presynaptic and postsynaptic mechanisms. Proc. Natl. Acad. Sci. USA *104*, 353–358. https://doi.org/10.1073/pnas.0608807104.

6. Wang, Z., Jackson, R.J., Hong, W., Taylor, W.M., Corbett, G.T., Moreno, A., Liu, W., Li, S., Frosch, M.P., Slutsky, I., et al. (2017). Human Brain-Derived Aβ Oligomers Bind to Synapses and Disrupt Synaptic Activity in a Manner That Requires APP. J. Neurosci. *37*, 11947–11966. https://doi.org/10.1523/JNEUROSCI.2009-17.2017.

7. He, Y., Wei, M., Wu, Y., Qin, H., Li, W., Ma, X., Cheng, J., Ren, J., Shen, Y., Chen, Z., et al. (2019). Amyloid β oligomers suppress excitatory transmitter release via presynaptic depletion of phosphatidylinositol-4,5-bisphosphate. Nat. Commun. *10*, 1193. https://doi.org/10.1038/s41467-019-09114-z.

8. Fogel, H., Frere, S., Segev, O., Bharill, S., Shapira, I., Gazit, N., O'Malley, T., Slomowitz, E., Berdichevsky, Y., Walsh, D.M., et al. (2014). APP Homodimers Transduce an Amyloid-β-Mediated Increase in Release Probability at Excitatory Synapses. Cell Rep. *7*, 1560–1576. https://doi.org/10.1016/j.celrep.2014.04.024.

9. Takahashi, R.H., Almeida, C.G., Kearney, P.F., Yu, F., Lin, M.T., Milner, T.A., and Gouras, G.K. (2004). Oligomerization of Alzheimer's β-Amyloid within Processes and Synapses of Cultured Neurons and Brain. J. Neurosci. *24*, 3592–3599. https://doi.org/10.1523/JNEUROSCI.5167-03.2004.

10. Snyder, E.M., Nong, Y., Almeida, C.G., Paul, S., Moran, T., Choi, E.Y., Nairn, A.C., Salter, M.W., Lombroso, P.J., Gouras, G.K., and Greengard, P. (2005). Regulation of NMDA receptor trafficking by amyloid-β. Nat. Neurosci. *8*, 1051–1058. https://doi.org/10.1038/nn1503.

11. Kurucu, H., Colom-Cadena, M., Davies, C., Wilkins, L., King, D., Rose, J., Tzioras, M., Tulloch, J.H., Smith, C., and Spires-Jones, T.L. (2022). Inhibitory synapse loss and accumulation of amyloid beta in inhibitory presynaptic terminals in Alzheimer's disease. Eur. J. Neurol. *29*, 1311–1323. https://doi.org/10.1111/ene.15043.

12. Willén, K., Sroka, A., Takahashi, R.H., and Gouras, G.K. (2017). Heterogeneous Association of Alzheimer's Disease-Linked Amyloid-β and Amyloid-β Protein Precursor with Synapses. J. Alzheimers Dis. *60*, 511–524. https://doi.org/10.3233/JAD-170262.

13. Selkoe, D.J. (2002). Alzheimer's Disease Is a Synaptic Failure. Science *298*, 789–791. https://doi.org/10.1126/science.1074069.

14. Meftah, S., and Gan, J. (2023). Alzheimer's disease as a synaptopathy: Evidence for dysfunction of synapses during disease progression. Front. Synaptic Neurosci. *15*, 1129036. https://doi.org/10.3389/fnsyn.2023.1129036.

15. Paula-Lima, A.C., Brito-Moreira, J., and Ferreira, S.T. (2013). Deregulation of excitatory neurotransmission underlying synapse failure in Alzheimer's disease. J. Neurochem. *126*, 191–202. https://doi.org/10.1111/jnc.12304.

16. Goutagny, R., and Krantic, S. (2013). Hippocampal oscillatory activity in Alzheimer's disease: toward the identification of early biomarkers? Aging Dis. *4*, 134–140.

17. Lauterborn, J.C., Scaduto, P., Cox, C.D., Schulmann, A., Lynch, G., Gall, C.M., Keene, C.D., and Limon, A. (2021). Increased excitatory to inhibitory synaptic ratio in parietal cortex samples from individuals with Alzheimer's disease. Nat. Commun. *12*, 2603. https://doi.org/10.1038/s41467-021-22742-8.

18. Targa Dias Anastacio, H., Matosin, N., and Ooi, L. (2022). Neuronal hyperexcitability in Alzheimer's disease: what are the drivers behind this aberrant phenotype? Transl. Psychiatry *12*, 257. https://doi.org/10.1038/s41398-022-02024-7.

19. Noebels, J. (2011). A perfect storm: Converging paths of epilepsy and Alzheimer's dementia intersect in the hippocampal formation. Epilepsia *52*, 39–46. https://doi.org/10.1111/j.1528-1167.2010.02909.x.

20. Ibrahim-Verbaas, C.A., Harold, D., Naj, A.C., Sims, R., Bellenguez, C., DeStafano, A.L., Bis, J.C., Beecham, G.W., and Grenier-Boley, B. (2013). Meta-analysis of 74,046 individuals identifies 11 new susceptibility loci for Alzheimer's disease. Nat. Genet. *45*, 1452–1458. https://doi.org/10.1038/ng.2802.

21. Seshadri, S., Fitzpatrick, A.L., Ikram, M.A., DeStefano, A.L., Gudnason, V., Boada, M., Bis, J.C., Smith, A.V., Carassquillo, M.M., Lambert, J.C., et al. (2010). Genome-wide Analysis of Genetic Loci Associated With Alzheimer Disease. JAMA *303*, 1832–1840. https://doi.org/10.1001/jama.2010.574.

22. Carnicero-Senabre, D., Barata, M.A., Jiménez-Villegas, J., Guimas Almeida, C., Cuadrado, A., and Rojo, A.I. (2025). NRF2 deficiency is associated with synaptic alterations and ether-linked phospholipid imbalance in the hippocampus. Redox Biol. *86*, 103853. https://doi.org/10.1016/j.redox.2025.103853.

23. Katrukha, E. (2020). Ekatrukha/ComDet: ComDet 0.5.3. Version 0.5.3. (Zenodo). https://doi.org/10.5281/ZENODO.4281064.