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Analyzing autophagosomes and mitophagosomes in the mouse brain using electron microscopy

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SUMMARY
Electron microscopy (EM) is considered the gold standard for studying macroautophagy and mitophagy, essential cellular processes for brain health. Here, we present a protocol using EM to analyze autophagosomes and mitophagosomes in the mouse amygdala. We describe the preparation of brain sections, followed by staining and EM imaging. We then detail the steps to identify and analyze autophagosome-like and mitophagosome-like structures. This protocol can be easily adapted to analyze autophagosomes and mitophagosomes in other mouse brain regions. For complete details on the use and execution of this protocol, please refer to Duan et al. (2021).

BEFORE YOU BEGIN
All animal work needs to be approved by the Institutional Animal Care and Use Committee prior to beginning the experiment. The laboratory needs to be equipped with a chemical fume hood and have a specifically designated and authorized location for handling and storing radioactive materials.

Preparation one: fixative

© Timing: 1 h

1. 0.4 M phosphate buffer (PB)
   a. Dissolve 56.0 g K$_2$HPO$_4$ and 10.6 g NaH$_2$PO$_4$ in Milli-Q or distilled water.
   b. Bring the volume to 1000 mL with Milli-Q or distilled water.
   c. Adjust pH to 7.4 with 1 M NaOH or 1 M HCl. Store at room temperature (20°C–22°C) indefinitely.

| Reagent   | Final concentration | Amount   |
|-----------|---------------------|----------|
| K$_2$HPO$_4$ | 0.322 M             | 56.0 g   |
| NaH$_2$PO$_4$ | 0.088 M             | 10.6 g   |
| H$_2$O     | n/a                 | Adjust to 1000 mL of total volume |
Note: The following solutions should be prepared on the day of perfusion.

2. 0.1 M PB with heparin
   a. Dilute 0.4 M PB to 0.1 M with Milli-Q or distilled water.
   b. Add heparin to a final concentration of 10 units/mL.

3. Fixative

Use 16% EM grade paraformaldehyde, 8% EM grade glutaraldehyde, and 0.4 M PB to prepare fixative containing 4% (v/v) paraformaldehyde, 2% (v/v) glutaraldehyde, and 0.1 M PB.

### Preparation two: 0.1 M cacodylate buffer

- **Timing:** 30 min

4. 1 M cacodylate stock solution
   a. Dissolve 42.8 g Na(CH₃)₂AsO₂·3H₂O in Milli-Q or distilled water.
   b. Bring the volume to 200 mL with Milli-Q or distilled water.
   c. Adjust to pH 7.4 with 0.2 M HCl. Store at room temperature (20°C–22°C) indefinitely.

### Preparation three: 0.3% lead citrate solution

- **Timing:** 30 min

5. 0.3% lead citrate solution
   a. Add 80 mL distilled water over 0.3 g lead citrate.
   b. Add 1 mL of 10 N NaOH and shake vigorously.
   c. Adjust volume to 100 mL with distilled water.
   d. Pass it through a 0.22 μm filter before use.
   e. Always keep the cap closed to minimize CO₂ contamination and store at 4°C. If the solution becomes white milky, it should be discarded.

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Chemicals, peptides, and recombinant proteins | | |
| Ketamine | VetOne | NDC 13985-584-10 |
| Xylazine | Akorn | NDC 59399-110-20 |
| Paraformaldehyde (16% aqueous) | Electron Microscopy Sciences | Cat#15710 |
| Glutaraldehyde (8% aqueous) | Electron Microscopy Sciences | Cat#16019 |
| Heparin | Sigma-Aldrich | Cat#9041-08-1 |
| Sodium cacodylate trihydrate | Sigma-Aldrich | Cat#C0250 |
| 4% osmium tetroxide stock solution | Electron Microscopy Sciences | Cat#19170 |
| Uranyl acetate | Electron Microscopy Sciences | Cat#22400 |
| Propylene oxide | Polysciences | Cat#00236 |

(Continued on next page)
Epon formula: Add all components in the order as they appear in the table. First, pour the EMBED812, DDSA, and NMA in order into a graduated 50 mL plastic centrifuge tube with a cap. Tighten the cap, then shake/rotate the tube gently by hand until the solution becomes homogeneous. Add the BDMA with a disposable pipette tip, then shake/rotate again to mix.

△ CRITICAL: Prepare fresh. Note that we also use some epon that has been stored at -20°C or -30°C in a sealed, 50 mL plastic centrifuge tube and warmed up to room temperature before use. Long-term cold storage may lead to partial polymerization, so it is only
recommended if you wish, for the step using a mixture of epon and propylene oxide. Also, instead of a plastic centrifuge tube for storage of epon, one could use a sealed syringe to exclude air, i.e., to prevent condensation that may affect resin polymerization.

⚠️ CRITICAL: Paraformaldehyde, glutaraldehyde, osmium tetroxide, uranyl acetate, BDMA, propylene oxide, EMBED 812, DDSA, and NMA are toxic. They are harmful if ingested, inhaled or in contact with skin. Uranyl acetate is radioactive. Propylene oxide is corrosive and a potential carcinogen. All these chemicals must be handled in a chemical fume hood by individuals wearing personal protection equipment (PPE) including at least lab coats and gloves (two layers of gloves for propylene oxide). All contaminated materials are to be disposed of as chemical waste or radioactive waste in compliance with regulations of hazardous waste.

Toluidine blue solution: Dissolve 500 mg toluidine blue and 1 g sodium tetraborate in 100 mL Milli-Q or distilled water.

| Reagent       | Final concentration | Amount  |
|---------------|---------------------|---------|
| Toluidine blue| 0.5% (w/v)          | 500 mg  |
| Sodium tetraborate | 1% (w/v)          | 1 g     |
| H₂O           | n/a                 | 100 mL  |

⚠️ CRITICAL: Wear lab coats and disposable gloves when preparing the toluidine blue solution. The toluidine blue solution can be stored at room temperature (20°C–22°C) indefinitely.

**STEP-BY-STEP METHOD DETAILS**

### Animal perfusion and brain sectioning

**Timing:** 2 days

The goal of this step is to fix the brain to preserve the structures of brains and organelles. High-quality perfusion is crucial for obtaining high-resolution electron micrographs. After perfusion, the brain is removed and cut with a vibratome into brain sections, which will be utilized in a later step to obtain ultrathin sections for EM. Care is taken during handling brain sections to avoid tissue damage.

1. **Animal perfusion**
   a. Conduct perfusion in a chemical fume hood. Use the perfusion buffer (0.1 M PB containing 10 units/mL heparin) and the fixative (4% paraformaldehyde and 2% glutaraldehyde in 0.1 M PB) at warm room temperature (23°C–37°C).
   i. Transcardially perfuse mice and extract the brains with a standard protocol. First, anesthetize mice with isoflurane and then inject them intraperitoneally with ketamine (110 mg/kg) and xylazine (7 mg/kg).
   ii. Wait until adequate anesthesia is achieved as determined by loss of response to toe pinching. Place the mouse on a shallow tray.
   iii. Make a small incision in the diaphragm beneath the rib cage using a pair of iris scissors.
   iv. Cut the diaphragm along the entire length of the rib cage to expose the pleural cavity.

   **Note:** Take care to avoid damaging the liver and the heart.

   v. Insert a blunt tip needle into the left ventricle of the heart.
   vi. Use a hemostat to clamp the heart to secure the needle and prevent leakage.
vii. Cut open the right atrium with a pair of iris scissors.

viii. Attach the needle base to a three-way valve connected to two 50-mL syringes, each containing 0.1 M PB with heparin or fixative.

ix. Turn the valve to open the PB line and manually inject 25 mL 0.1 M PB with heparin within 1 min.

*Note:* The liquid coming out of the atrium should be clear and the lungs and liver should have become very light after perfusion with PB.

x. Switch the perfusion solution to fixative and manually inject 25 mL fixative within ~4 min.

b. Isolate the brain.

i. Remove the head with a pair of scissors.

ii. Expose the skull by making an incision along the midline from the neck to the nose. Remove the vertebrae and muscles that remain attached to the skull.

iii. Use the tip of a pair of iris scissors to carefully cut open the skull. Lift up the blade when cutting to prevent damaging the brain. Use a rongeur to peel the skull away from the dorsal surface of the brain.

iv. Use a spatula to sever the olfactory bulbs and nerves connected to the ventral surface of the brain.

c. Place the isolated brain in fresh fixative overnight (12–18 h) at 4°C.

2. Brain sectioning

a. Prepare a 5% agarose block. Add 5 g agarose powder and 0.85 g NaCl to 100 mL Milli-Q or distilled water. Heat the solution in a microwave oven until boiling. Pour the agarose solution into several 10-cm tissue culture plates and let it cool down.

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| Agarose | 5% (w/v)            | 5 g    |
| NaCl    | 0.85% (w/v)        | 0.85 g |
| H2O     | n/a                 | 100 mL |

b. Cut the agarose block into 3 × 1 × 1.5 cm (L × W × H) cubes.

c. After the overnight fixation, wash brains with PBS (3×20 min). Gently agitate brains on a rotating platform during incubation with PBS. Alternatively, PB may be used; PBS is used because it works as well for this step and is readily available in the laboratory. Note that during Step 2, keep the brain wet with PBS or PB at all times to avoid any drying artifacts.

d. Glue the agarose cube to the sample holder of the vibratome with Krazy glue (Elmer’s Products, Westerville, OH). Extend the Krazy glue at the holder plate to avoid any drying artifacts.

e. Make a clean, vertical cut along the rostral border of the cerebellum to remove the cerebellum, using a razor blade.

f. Place the brain on a piece of filter paper to dry the bottom surface of the brain; this surface should be only slightly moist to ensure that the glue sticks properly.

g. Glue the brain without the cerebellum to the sample holder of the vibratome against the agarose block using Krazy glue. Orient the brain with the dorsoventral axis in parallel with the holder plate and the rostral tip of the brain facing up.

h. Fill the cutting chamber of the vibratome with PBS. Cut 150-μm thick coronal sections. Transfer brain sections into 6-well plates with a fine brush.

i. Wash the brain sections with 0.1 M cacodylate buffer diluted from the stock solution (3×5 min), then proceed to Step 3 directly if time permits. Otherwise, keep brain sections at 4°C overnight (12–18 h) and conduct Step 3 on the next day.
Osmium tetroxide fixation, en bloc staining, dehydration, and resin infiltration

© Timing: 1–1.5 days

Osmium tetroxide (OsO₄) is a secondary fixative for electron microscopy; it also provides contrast staining. Uranyl acetate is used for contrast staining for electron microscopy; it also acts as a fixative. The brain sections are dehydrated with ethanol following secondary fixation and staining, then embedded in epon resins. Steps 3–4 need to be performed in a chemical fume hood.

3. Osmium tetroxide fixation
   a. Prepare fresh 1% osmium tetroxide solution by adding 250 µL 4% osmium stock solution and 100 µL 1 M cacodylate stock solution to Milli-Q or distilled water. Bring the volume to 1 mL.
      b. Remove cacodylate buffer from brain sections by using a disposable transfer pipette. Do not allow the brain sections to dry during any of the procedures in Step 3.
         i. Add 1 mL or at least enough freshly made 1% osmium tetroxide solution to cover the sections, using a fresh pipette.
         ii. Incubate brain sections with the osmium tetroxide solution at room temperature (20°C–22°C) for 30 min in the dark.

Note: Excessive light and heat may alter osmium tetroxide and reduce its effectiveness (Hayat, 2000; Woods and Stirling, 2008). Thus, we fix the tissue in the dark for a short time at room temperature; we prefer room temperature since osmium tetroxide penetrates the tissue slowly, and cooling might slow penetration even more. Alternatively, one can fix for longer in both dark and cold. However, it is not clear if light versus dark can make a significant difference considering these relatively short incubation times.
   c. Wash brain sections 3×10 min with 0.1 M cacodylate buffer.

4. Dehydration and resin infiltration
   a. Prepare 1% uranyl acetate solution by adding 0.01 g uranyl acetate to 1 mL of 50% ethanol. Protect the 1% uranyl acetate solution from light.
   b. Dehydrate the brain sections with 50% ethanol (3×5 min).
   c. Replace 50% ethanol with the 1% uranyl acetate in ethanol solution. Keep brain sections in the 1% uranyl acetate solution for 15 min in the dark.
   d. Continue to dehydrate brain sections with 75% ethanol (2×5 min), 95% ethanol (10 min), and 100% ethanol (3×10 min).
   e. Carefully transfer brain sections to 20 mL glass scintillation vials containing propylene oxide. After transfer, change propylene oxide once.
   f. Replace propylene oxide with a 1:1 mixture of epon and propylene oxide. Incubate brain sections with the mixture for 1 h.
   g. Replace the epon and propylene oxide mixture with epon. Keep brain sections in epon at room temperature (20°C–22°C) overnight (12–18 h).
   h. On the day after dehydration, replace the overnight epon with freshly-made epon. Keep brain sections in epon for several hours (we keep them for 24 h) at room temperature (20°C–22°C).

| Reagent              | Final concentration | Amount  |
|----------------------|---------------------|---------|
| 4% osmium stock solution | 1% (v/v)           | 250 µL  |
| 1 M cacodylate       | 0.1 M               | 100 µL  |
| H₂O                  | n/a                 | 650 µL  |
| **Total**            | n/a                 | 1 mL    |

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**Embedding, isolation and mounting of the amygdala**

- **Timing:** 3–4 days

This step is to prepare tissue blocks that will be cut into ultrathin sections. The researcher identifies the brain regions of interest under a light microscope and uses a scalpel to isolate them. The isolated epon-embedded tissues are mounted on cylinders made of epon.

5. Tissue embedding and isolation
   a. Place dehydrated, epon infiltrated tissues between two Aclar sheets separated by a thin layer of epon to make an Aclar sandwich. Avoid trapping bubbles in the sandwich and cure it in an oven set at 64°C for 48 h to complete the embedding procedure.
   b. Additional epon can be used to make the epon cylinders. Mount flat bottom embedding capsules (polyethylene; size “00”; Electron Microscopy Sciences, catalog number 70021) on a corresponding polypropylene capsule holder (6 pack from EMS; catalog number 70022-06). Pour the epon into the capsules and heat in an oven as above.
   c. Remove the Aclar sandwich from the oven and cool it down at room temperature.
   d. Remove one of the Aclar sheets. Then, under a light microscope, outline the amygdala with a marker pen; this area can be identified by comparing its shape and features to those seen in coronal sections in any standard atlas. Use a scalpel to carve out the amygdala along the marked outlines.

6. Tissue mounting
   a. Prior to mounting tissues, use a razor blade to slightly scratch one end of the epon cylinder in a grid pattern to assist in adhesion of the tissue section.
   b. Glue the epon-embedded amygdala to the center of the grid with one drop of Krazy glue placed on the side still covered with the Aclar material.

**Note:** Ensure the tissue glued to the cylinder is flat and avoid using excessive glue. This usually produces a stable specimen for utrasectioning; however, if the tissue comes loose from the Aclar sheet, then the Aclar sheet can be removed and the tissue mounted directly to the cylinder.

   c. Dry the cylinder mounted with the tissue at room temperature overnight (12–18 h).
   d. Use a specimen holder supplied with the Ultramicrotome to keep the epon cylinder mounted with brain tissue in place and free both hands for trimming.
   e. Trim the edges of the tissue to make it a trapezoid. Don’t damage the tissue.
   f. Wipe off any debris from the cylinder with wet paper towels. Since the debris may contain stained tissue with heavy metals, it is preferred to wipe it off instead of blowing it off into the air.

**△ CRITICAL:** Use wood handles derived from cotton swabs to transfer epon-embedded tissues onto Aclar sheets. After the tissue is flattened on the first Aclar sheet, place one end of the second Aclar sheet on the first sheet and slowly lay down the rest of the second sheet to avoid bubbles. Illustrations of the epon cylinder and Aclar sandwich are shown in Figure 1.

**Cutting ultrathin brain sections**

- **Timing:** 8 h

7. Cut thick brain sections
a. Cut thick sections (1 μm or more) from the face of the mounted brain tissue block on an ultramicrotome until the entire tissue face is exposed. We use a Diatome Histo Diamond Knife (45° angle, 6 mm; catalog number 60-HIS; Diatome U.S., Hatfield, PA; affiliated with EMS).

b. Stain these sections with a toluidine blue solution (0.5% toluidine blue [500 mg] plus 1% sodium tetraborate [1 g in 100 mL water]); we stain them on a hot plate and continue until the solution is dried.

c. Examine these thick sections under a light microscope to determine if the proper area has been reached for electron microscope examination, i.e., comparing the area to the known features as shown in a standard atlas.

8. Cut ultrathin brain sections

a. Cut ultrathin brain sections (60 nm) from the face of the mounted brain tissue with an ultramicrotome. We use a Diatome Ultra Diamond Knife (Wet; 45° angle, 3.5 mm; catalog number 35-US).

b. Mount the ultrathin sections on formvar/carbon-coated single-slot copper grids (EMS, catalog number FCF2010-Cu-SB). Alternatively, nickel grids (EMS, catalog number FCF2010-Ni-SB) may also be used.

c. Store grids in a grid box (EMS, catalog number 71147-12) in a desiccator until use.

The ultrathin brain section is shown in Figure 2.

**Electron microscope imaging**

© Timing: 4–8 h

9. Staining

a. We mount the grids on a Hiraoka grid support plate. The type that we use has been discontinued (see Figure 1 in Petralia and Wang, 2021), but a similar, modified Hiraoka grid support
plate is available separately (EMS, catalog number 71560-32) or in the Modified Hiraoka Staining Kit (EMS, catalog number 71560-00).

b. Stain with 0.3% lead citrate solution (0.3 g lead citrate in 0.1 N NaOH; pass it through a 0.22 μm filter before use) for 3 min and then wash 3× with distilled water. Dry using a pipette to remove most of the water and then use the edge of a piece of filter paper to remove the last traces of water. Store in grid box until use.

10. Electron microscope imaging
   a. Place a grid with a section of the brain in a holder and insert it into the JEOL JEM-2100 transmission electron microscope (TEM).
   b. Align and examine at 200 kV.
Note: This moderately high voltage allows electrons to penetrate the tissue more readily to improve image quality; and the JEOL 2100 maintains high image contrast at 200 kV as long as there is optimal sample preparation and thickness, due to its pole-piece with an in-gap, high-contrast objective lens aperture. Our lab and others routinely use the JEOL 2100 at 200 kV for all types of studies. For some other TEMs, one might need to use a lower kV to ensure adequate contrast.

c. Go to low magnification (50–100×) and compare the image on the Gatan camera monitor screen (using Digital Micrograph program) with an image of the last 1 μm section taken before the ultrathin sections (see Figure 2). Then mark off the boundaries of the brain region on the ultrathin section to be imaged, using the JEOL specimen position display window.

d. Take images at high magnifications (2,500× to 25,000×).

EXPECTED OUTCOMES

Every attempt is made to acquire an unbiased sample for quantitative analysis. During ultramicrotomy, we must take the thin sections from a very restricted and relatively small area in order to identify the boundaries of the amygdala nuclei accurately, so we are not able to utilize a wide area of the tissue to acquire random samples. However, the thin sections on grids from the different mice are assigned a code number unknown to the person who will image them on the TEM, so that the EM images are taken blindly. The imager marks off the boundaries of the relatively small area of the particular amygdala nucleus and then selects random areas within those boundaries only at low magnifications, so that the subcellular structures of interest cannot be discerned. Then, the imager goes to high magnifications on each of these randomly selected areas and captures the high-magnification images used in the study.

Electron micrographs are obtained after image acquisition and saved in the DM3 or DM4 format. The DM3/DM4 files are converted to JPEG files for image analysis. Subcellular structures can be resolved from electron micrographs at nanometer-resolutions. Autophagosomes and mitophagosomes are identified manually from electron micrographs. Subcellular structures with electron-dense contents and bound by a double limiting membrane are identified as autophagosome-like structures. Of these structures, those containing discernible mitochondria, as indicated by the presence of double membranes and presumptive cristae, are identified as putative mitophagosome structures; we call them mitophagosome-like structures to indicate that the identification of the cristae is not always definitive. Examples of autophagosome-like and mitophagosome-like structures are shown in Figure 3.

QUANTIFICATION AND STATISTICAL ANALYSIS

The experimenter is blind to the identity of the electron micrographs. The numbers of autophagosomes and mitophagosomes in each micrograph are counted manually. The total area of each micrograph is measured with commonly used image analysis software such as ImageJ, Metamorph, and Imaris. We count all autophagosome-like and mitophagosome-like structures in each micrograph and analyze all micrographs including those without autophagosome-like or mitophagosome-like structures in which the numbers of such structures are recorded as “0”. Micrographs at the same magnification are used for quantitative analysis. The density of autophagosome-like and mitophagosome-like structures is calculated by dividing their total numbers in each micrograph by the area of the micrograph and averaged over ~200 micrographs. Appropriate statistical analysis is conducted if the study goal is to determine the effect of experimental treatment on autophagosomes and mitophagosomes. Specific statistical tests are chosen based on the experimental design, number of experimental conditions, number of variables, sample size, and the null hypothesis to be tested.

LIMITATIONS

Autophagosomes and mitophagosomes are transient structures that are sensitive to the extracellular and intracellular environments affected by the animal’s experience such as housing conditions, anesthesia, and behavioral testing. The quality of electron microscopy is dependent on the procedures of preparing brain tissues including animal perfusion, tissue fixation, and tissue staining. These
biological and technical factors may have significant effect on the sensitivity and quantifiability of the method described in this protocol. Careful consideration of control groups, minimization of stress to the animal, standardization of animal housing, handling and behavioral testing, and quality check for each step of tissue preparation are warranted to achieve high-quality results. The numbers of autophagosomes and mitophagosomes in each cell are relatively small. An adequate number of images and size of imaging fields are necessary to obtain reproducible results for both discovery and quantitative studies. We typically collect ~200 micrographs (5.6 × 3.38 μm) from 3 animals for each group. Because this protocol relies on visual inspection to identify mitophagosome-like and autophagosome-like structures, the data analysis step is time-consuming and needs experienced researchers proficient at identifying these structures from electron micrographs. These factors limit the throughput and efficiency of the EM-based analysis of macroautophagy and mitophagy.

**TROUBLESHOOTING**

**Problem 1**
Ultrastructural examination shows a lack of microtubules (step 1).
**Potential solution**
When fixing the tissue, make sure that the fixative initially is at room temperature or warmer to avoid loss of microtubules that may occur with initial fixation in cold fixative.

**Problem 2**
Poor ultrastructure including membrane damage and swollen or shrunken organelles (steps 1 and 3).

**Potential solution**
Keep the perfusion wash-out time short (less than 1 min) prior to adding the fixative to avoid loss of ultrastructure quality. Proper osmolality and pH of the fixative are also important to avoid swelling or shrinkage of organelles. It is best to use only sealed aliquots of paraformaldehyde and glutaraldehyde and make up the fixative just before use. The same is true for the osmium tetroxide solution. It should be a light color before use. If it is dark, it is likely bad and should not be used. The tissue should look evenly dark when the osmium tetroxide step is completed; if not, one may need to agitate the solution during this step. Poor osmium fixation can affect both tissue ultrastructure and contrast (see next problem). Make sure that the tissue is never allowed to dry during any of the solution changes in the fixation, sectioning, and dehydration procedures.

**Problem 3**
Sections under TEM show poor contrast (steps 9 and 10).

**Potential solution**
This could be due either to problems in section preparation or TEM operation. You might want to make up a new lead citrate solution. When making it, we generally keep the container closed at all times of the mixing, although it is not clear if this is absolutely necessary. We sometimes add an additional step of staining with 1% uranyl acetate for 5 min (followed by washing and drying) prior to staining with lead citrate. In the TEM, one can try using a smaller objective aperture. We noted that using JEOL 2100 at 200 kV gives good contrast, but those using other TEMs may need to use lower kVs such as 80–100.

**Problem 4**
Sections under TEM are contaminated with particles of various sizes and shapes (step 9).

**Potential solution**
In order to avoid lead contamination artifacts on the section, when staining with lead citrate, be careful not to allow any partial drying while washing with water. Also, make sure that the diamond knife and water trough on the ultramicrotome and all instruments are clean.

**Problem 5**
Borders of the desired brain region are unclear in the TEM (step 10).

**Potential solution**
One may overcome difficulties with setting the exact areas of brain regions (such as nuclei within the amygdala) by looking for clear markers such as blood vessels and irregular features visible on the thick section that is used to orient/correlate with the low magnification image seen with the TEM camera.

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Zheng Li (lizheng2@mail.nih.gov).

**Materials availability**
This study did not generate new materials.
Data and code availability
This study did not generate new data or code.

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AUTHOR CONTRIBUTIONS
All authors wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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