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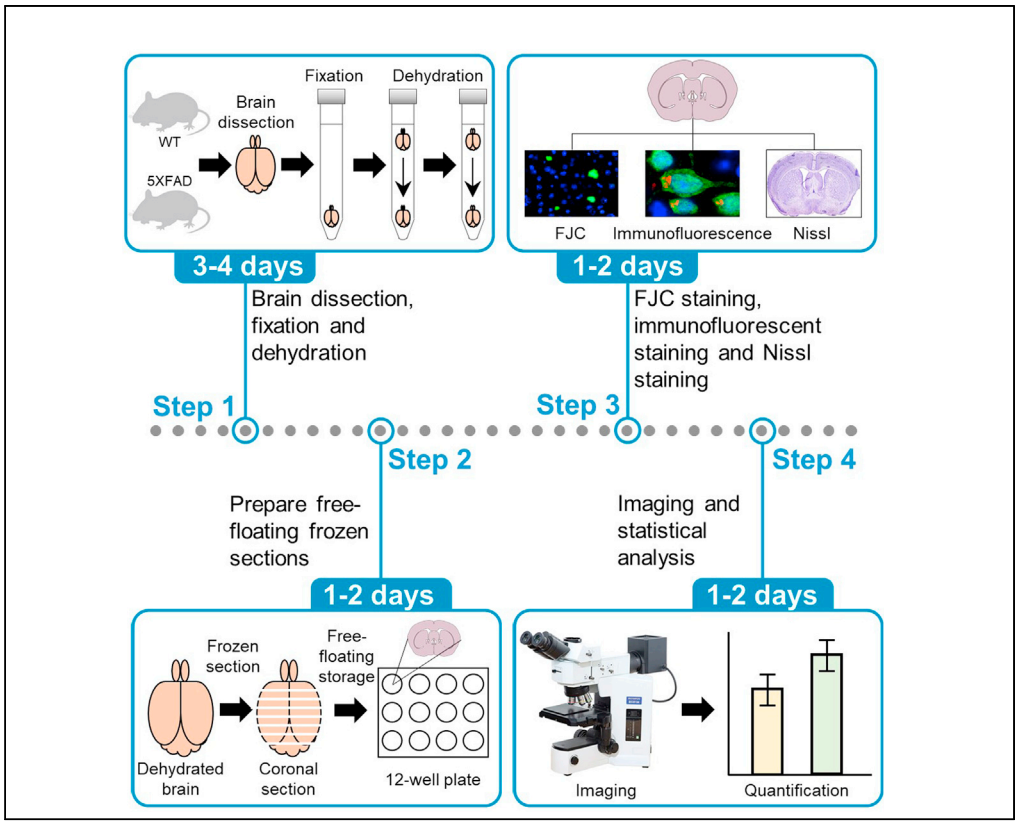
Assessment of neurodegeneration and neuronal loss in aged 5XFAD mice



Neuronal loss resulting from progressive neurodegeneration is a major pathological feature of Alzheimer's disease (AD). Here, we present a protocol to detect neurodegeneration, neuronal apoptosis, and neuronal loss in 5XFAD mouse strain, which is a well-established model for interrogating the molecular mechanism of neuronal death in AD. This protocol describes the use of the neurodegenerative marker Fluro-Jade C, cleaved caspase-3 immunofluorescent staining, and Nissl staining for the analysis of neurodegeneration and neuronal loss in 5XFAD mice.

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Highlights

Aged 5XFAD mice exhibit neurodegeneration, neuroapoptosis, and neuronal loss

Analysis of neurodegeneration with the neurodegenerative marker Fluro-Jade C

Detection of apoptotic neurons by cleaved caspase-3 immunofluorescent staining

Assessment of neuronal loss by Nissl staining

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Assessment of neurodegeneration and neuronal loss in aged 5XFAD mice

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SUMMARY

Neuronal loss resulting from progressive neurodegeneration is a major pathological feature of Alzheimer's disease (AD). Here, we present a protocol to detect neurodegeneration, neuronal apoptosis, and neuronal loss in 5XFAD mouse strain, which is a well-established model for interrogating the molecular mechanism of neuronal death in AD. This protocol describes the use of the neurodegenerative marker Fluro-Jade C, cleaved caspase-3 immunofluorescent staining and Nissl staining for the analysis of neurodegeneration and neuronal loss in 5XFAD mice.

For complete details on the use and execution of this protocol, please refer to Zhang et al. (2021).

BEFORE YOU BEGIN

This protocol describes the specific steps to analysis (1) neurodegeneration with the method of Fluro-Jade C (FJC) staining, (2) neuronal apoptosis using immunofluorescent staining with anti-cleaved caspase-3 antibody and (3) neuronal loss using Nissl staining combined with immunofluorescent staining with anti-NeuN antibody, in the brain slices of 9-month-old 5XFAD mice and age-matched wide-type (WT) control mice. In our experience, neurodegeneration can be analyzed using 6–9 months old 5XFAD mice. However, using 9–12 months old 5XFAD for neuronal apoptosis and neuronal loss analysis would be better, because the signal of cleaved caspase-3 is hard to be detected in the brain of younger 5XFAD mice.

Before the staining, you need to prepare the following animals and solutions (refer to ''Materials and equipment'' for detailed recipe.):

9 months old 5XFAD male mice and WT control male mice.

Pre-chilled 1× PBS for the perfusion of mouse brains.

4% paraformaldehyde (PFA) fixing solution.

Anti-freezing solution for the storage of mouse brain sections.

0.01% Fluro-Jade C stock solution for FJC staining.

0.06% potassium permanganate solution for FJC staining.

80% ethanol containing 1% NaOH for FJC staining.

Blocking buffer for immunofluorescent staining.

PBST washing buffer for immunofluorescent staining.

0.5% Cresyl violet solution for Nissl staining.

95% ethanol containing 0.7% glacial acetic acid de-staining solution for Nissl staining.

Prepare coronal sections

Timing: 4–5 days

1. Brain samples preparation
   a. Anesthetize mice with Ketamine/Xylazine (Ketamine: 50–80 mg/kg; Xylazine: 5–10 mg/kg) via intraperitoneal injection. Toe pinch reflection is recommended to monitor the depth of anesthesia. It usually takes 5–10 min to complete anesthesia.
   b. Perfuse the mice with ice-cold 1× PBS via left ventricle.
   c. Dissect out the whole brain carefully, and then put the brain into a 15 mL centrifuge tube containing 4% PFA over night with gentle shaking on an orbital shaker at a speed of 50 rpm in cold room (4°C–8°C).

2. Dehydrate the brain samples
   a. Next day, prepare 15% sucrose solution with 1× PBS freshly, then transfer the fixed brain to a 15 mL centrifuge tube containing 15% sucrose solution, keep the tube standing in cold room until the brain falls to the bottom of the tube, it usually takes 1 day.
   b. After the brain has fallen, typically on the third day since beginning the protocol, transfer the brain to another 15 mL centrifuge tube containing freshly prepared 30% sucrose dissolved in 1× PBS, still keep the tube standing in cold room until the brain falls to the bottom of the tube, this step will take 1–2 days.

   Note: Use filtered or autoclaved 1× PBS solution with 0.1% sodium azide to prepare sucrose solutions for long-term storage of the brain.

   c. Store dehydrated brain at −80°C until ready to prepare frozen sections.

   Note: The dehydrated brain can be stored at −80°C for 1 year.

3. Make brain coronal sections
   a. Section dehydrated brains for serial 30–50 μm coronal sections using a Thermo NX50 freezing microtome.
   b. Store coronal section in anti-freezing solution at −20°C before staining.

   Note: Both mounted and free-floating sections are suitable for the following staining procedures. Free-floating sections are used in the following protocols.

   ⚠ CRITICAL: The usage of Ketamine/Xylazine anesthetic and the performance on mice should conform to the relevant regulatory standards of Institutional Animal Care and Use Committee (IACUC).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-Cleaved caspase-3 (1:100)	Millipore	Cat# AB3623; RRID:AB_91556
Guinea pig anti-NeuN (1:500)	Millipore	Cat# ABN90P; RRID:AB_2341095
Donkey anti-rabbit IgG (H+L) Alexa Fluor® 594 (1:1000)	Jackson ImmunoResearch	Cat# 711-585-152 RRID:AB_2340621
Donkey anti-guinea pig IgG (H+L) Alexa Fluor® 488 (1:1000)	Jackson ImmunoResearch	Cat# 706-545-148 RRID:AB_2340472
Chemicals, peptides, and recombinant proteins		
Fluoro-Jade C	Millipore	Cat# AG325
Cresyl Violet	Sigma-Aldrich	Cat# C5042
DAPI	Sigma-Aldrich	Cat# D9542
Sucrose	Sigma-Aldrich	Cat# V900116
PVP-40	Sigma-Aldrich	Cat# PVP40
Ethylene Glycol	Sigma-Aldrich	Cat# 324558
Glacial acetic acid	Sigma-Aldrich	Cat# A6283
Potassium permanganate	Sigma-Aldrich	Cat# 31404
NaOH	Sigma-Aldrich	Cat# 221465
PBS	Sigma-Aldrich	Cat# P3813
Tween 20	Sigma-Aldrich	Cat# P1379
Tissue-Tek O.C.T. Compound	Sakura	Cat# 4583
Normal Donkey Serum	Jackson ImmunoResearch	Cat# 071-000-121
Neutral balsam	Solarbio	Cat# G8590
16% Formaldehyde	Cell Signaling Technology	Cat# 12606
Ketamine	R&D	Cat# 3131/50
Xylazine	Selleck	Cat# S2516
Experimental models: Organisms/strains		
Mouse: B6.Cg-Tg(APPSwFlLon,PSEN1* M146L*L286V)6799Vas/Mmjax (5XFAD)	The Jackson Laboratory	Cat# 34848-JAX; RRID: MMRRC_034848-JAX
Mouse: C57BL/6J	The Jackson Laboratory	Cat# JAX:000664; RRID:IMSR_JAX:000664
Software and algorithms		
Fiji-ImageJ	National Institute of Health	https://imagej.net/Fiji; RRID:SCR_003070
GraphPad Prism 6	GraphPad	https://www.graphpad.com/scientific-software/prism/; RRID:SCR_002798
Other		
Olympus BX51 microscope	Olympus	BX51
Leica TCS SP8 confocal microscope	Leica	SP8
Thermo NX50 freezing microtome	Thermo Fisher Scientific	NX50
Gelatin-coated microscope slides	FD Neurotechnologies	Cat# PO101
Fluorescence Mounting Medium	Dako	Cat# S3023

MATERIALS AND EQUIPMENT

- 4% PFA fixing solution:

Reagent	Final concentration	Amount or volume
16% Formaldehyde	4%	10 mL
1× PBS	n/a	30 mL
Total	**n/a**	**40 mL**

Note: 4% PFA solution need to be prepared freshly.

- Anti-freezing solution:

Reagent	Final concentration	Amount or volume
$NaH_2PO_4 \cdot 2H_2O$	0.02 M	1.560 g
$Na_2HPO_4 \cdot 12H_2O$	0.08 M	3.581 g
PVP-40	1%	5 g
Sucrose	30%	150 g
Ethylene glycol	30%	150 mL
ddH_2O	n/a	To 500 mL
Total	**n/a**	**500 mL**

Note: Anti-freezing solution can be stored at room temperature (20°C–25°C) up to 2 weeks.

- 0.06% potassium permanganate:

Reagent	Final concentration	Amount or volume
Potassium permanganate	0.06%	0.015 g
ddH_2O	n/a	To 25 mL
Total	**n/a**	**25 mL**

Note: 0.06% potassium permanganate solution need to be prepared freshly.

- Blocking buffer:

Reagent	Final concentration	Amount or volume
Normal donkey serum	5%	0.5 mL
BSA	1%	0.1 g
1× PBS	n/a	To 10 mL
Total	**n/a**	**10 mL**

Note: Blocking buffer can be stored at 4°C up to 2 days.

- PBST washing buffer:

Reagent	Final concentration	Amount or volume
Tween 20	0.2%	0.1 mL
1× PBS	n/a	To 50 mL
Total	**n/a**	**50 mL**

Note: Washing buffer can be stored at room temperature up to 1 week.

- De-staining solution:

Reagent	Final concentration	Amount or volume
Glacial acetic acid	0.7%	1.4 mL
100% ethanol	95%	190 mL
ddH_2O	n/a	8.6 mL
Total	**n/a**	**200 mL**

Note: De-staining solution can be stored at room temperature for 1 month.

- 80% ethanol containing 1% NaOH:
  - Dissolve 0.25 g NaOH with 5 mL distilled water.
  - Mix above NaOH solution with 20 mL 100% ethanol.
  - Store this solution at room temperature for up to 1 day.
- Fluro-Jade C staining solution:
  - Dissolve 10 mg Fluro-Jade C in 100 mL distilled water to make 0.01% stock solution.
  - Dilute 0.1 mL glacial acetic acid with 100 mL distilled water to make 0.1% acetic acid solution.
  - Just before use, dilute 0.01% FJC stock solution in 0.1% acetic acid solution to make 0.0001% working solution with a ratio of 1:100.
  - Store 0.01% FJC stock solution and 0.1% acetic acid solution at 2°C–8°C for up to 3 months.
- Cresyl violet solution:
  - Dissolve 1 g Cresyl violet acetate in 200 mL distilled water.
  - Add 500 μL glacial acetic acid and filter the solution with filter paper.
  - Store 0.5% Cresyl violet solution at room temperature for up to 6 months.

⚠ CRITICAL: Glacial acetic acid and NaOH are corrosive, and PFA is toxic in contact with skin and if inhaled. Always wear gloves, mask and lab coat to protect yourself, and handle glacial acetic acid and PFA under a chemical hood.

Alternatives: In principle, the reagents and resources listed in the key resources table can be replaced with equivalent items from other suppliers; however, the impact of alternative reagents on protocol performance has not been tested.

Alternatives: This protocol uses Olympus BX-51 microscope and Leica TCS SP8 confocal microscope for imaging. Alternative microscopes can be used as well.

STEP-BY-STEP METHOD DETAILS

Fluro-Jade C staining of degenerated neurons

Timing: 2–3 h

Rationale: Fluro-Jade C staining is able to stain all degenerating neurons, regardless of specific insult or mechanism of cell death. It is ideal for localizing not only degenerating nerve cell bodies, but also distal dendrites, axons and terminals, due to greatest signal to background ratio, as well as the highest resolution. Sections labeled with FJC can also be immuno-stained to achieve multiplexed labeling.

1. Pre-treating free-floating brain sections
   a. Wash free-floating brain sections with 1× PBS 5 times, 5 min for each time, to remove anti-freezing solution completely.
   b. Pre-treat sections as indicated below:

Step	Solution	Time of immersion
1	80% ethanol containing 1% NaOH	5 min
2	70% ethanol	2 min
3	50% ethanol	2 min
4	30% ethanol	2 min
5	10% ethanol	2 min
6	ddH_2O	2 min, twice
7	0.06% potassium permanganate	10 min
8	ddH_2O	2 min, twice

Note: Pre-treating should be performed in the wells of a 24- or 12-well cell culture plate for free-floating brain sections. Brain sections will shrink in 0.06% potassium permanganate solution and expand in ddH_2O, but will recover to normal shape in PBS. Keep staining regardless of shape change of the brain sections.

2. Performing FJC staining
   a. Dilute DAPI stock solution (0.1 mg/mL) in 0.0001% FJC working solution at a ratio of 1:1000.
   b. Stain brain sections with 0.0001% FJC working solution containing DAPI dye for 20 min.
   c. Wash brain sections with ddH_2O for 3 times to remove extra FJC solution.
   d. Recover brain sections in 1× PBS.

Pause point: Sections can be stored in 1× PBS for 0.5–1 h before mounting.

   e. Mount brain sections on slides with Fluorescence Mounting Medium.
   f. Put cover glass on the slides and push off bubbles and remove extra mounting medium.
   g. Dry the mounted slides in the dark overnight (12–14 h) at room temperature.

Note: This protocol refers to a wet mount media, and nail polish should be used to seal coverslips. The mounted slides can be stored at 4°C for 1 month before imaging.

3. Quantification of degenerated neurons
   a. Take pictures of degenerated neurons in cortex and hippocampus using an Olympus BX51 microscope with 20× objective lens (Figure 1). Use filter cube WU-#1 (dichroic mirror DM400, excitation filter BP330-385, barrier filter BA420) to visualize DAPI, and use filter cube WB - #2 (dichroic mirror DM500, excitation filter BP450-480, barrier filter BA515) to visualize FJC. For each mouse brain sample, three to five serial sections should be stained, and entire brain region should be pictured for quantification.
   b. Open pictures with Fiji-ImageJ software, and use modules of "Measure" and "Analyze Particles" to calculate the density of degenerated neurons by measuring the brain area and counting the number of degenerated neurons respectively.
   c. Statistically analyze the density (the number of FJC-positive neurons in given brain area (mm^2)) of degenerated neurons among several groups using GraphPad Prism 6 software.

Immunofluorescent staining of apoptotic neurons

Timing: 2 days

Rationale: Apoptotic cells have the feature of enhanced activation of caspase-3, which is a specific executioner caspase in apoptosis. Anti-NeuN and anti-cleaved caspase-3 double staining is able to detect apoptosis specifically in neurons.

4. Wash brain sections with 1× PBS for 3 times, 10 min for each time, to thoroughly remove anti-freezing solution.
5. Permeabilize sections in 0.3% Triton X-100 in 1× PBS for 30 min.
6. Incubate sections in blocking buffer (5% normal donkey serum and 1% BSA in 1× PBS) for 1 h at room temperature.
7. Prepare primary antibody dilutions of anti-cleaved caspase-3 (1:100) and anti-NeuN (1:500) with blocking buffer.
8. Incubate sections in primary antibodies overnight (12–14 h) on an orbital shaker at a speed of 50 rpm at 4°C.
9. Next day, wash sections with PBST washing buffer for 3 times, 10 min for each time.

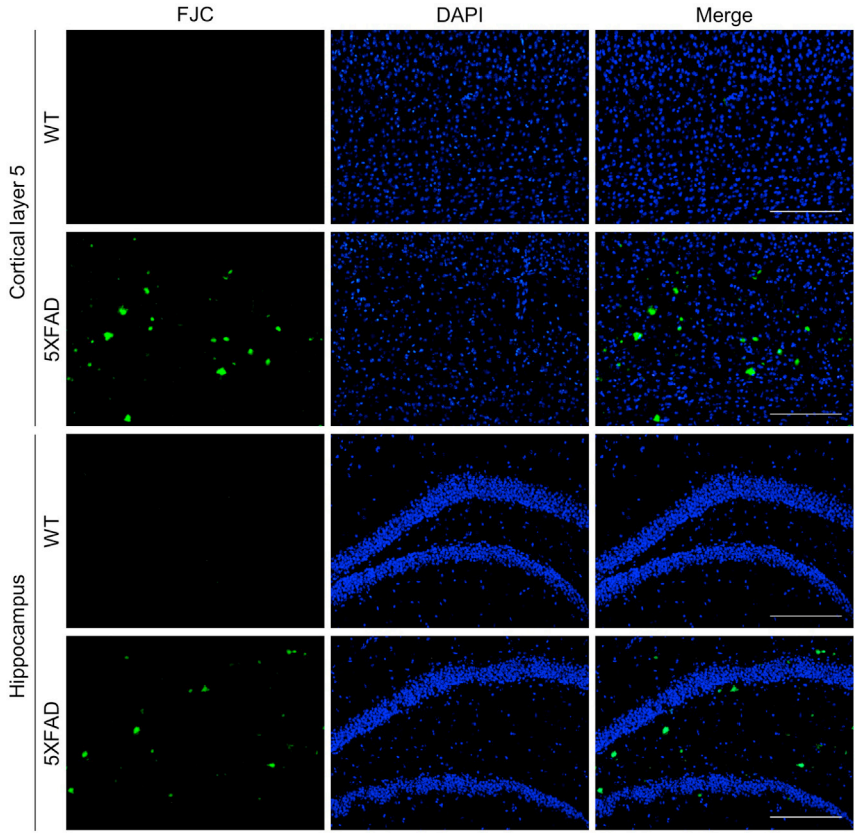

Figure 1. Degenerated neurons detected by FJC staining in aged 5XFAD mice

Representative pictures of degenerated neurons labeled by FJC in cortex and hippocampus of 9-month-old WT and 5XFAD mice. Scale bar, 200 μm.

10. Prepare secondary antibody solutions of donkey anti-rabbit IgG Alexa Fluor® 594 (1:1000) and donkey anti-guinea pig Alexa Fluor® 488 (1:1000), or fluorophores of your choice, with blocking buffer.
11. Incubate sections with secondary antibodies for 2 h at room temperature to visualize primary antibodies.
12. Wash sections with PBST for 3 times, 10 min for each time.
13. Incubate sections with 100 ng/mL DAPI solution to stain cell nuclei.
14. Mount brain sections on slides with Fluorescence Mounting Medium.
15. Put cover glass on the slides and push off bubbles and remove extra mounting medium.
16. Dry the mounted slides in the dark overnight (12–14 h) at room temperature.

Note: This protocol refers to a wet mount media, and nail polish should be used to seal coverslips. The mounted slides can be stored at 4°C for 1 month before imaging.

17. Image cleaved caspase-3 positive neurons using a Leica TCS SP8 confocal microscope (Figure 2). Typically, three to five serial sections per mice, and four to six fields per section need to be randomly pictured for quantification. 40X or 63X objective lens is recommended for imaging. Excitation and emission filters are used based on the fluorophores of your choice.
18. Statistically analyze the ratio of apoptotic neurons (number of apoptotic neurons/number of total neurons) among several groups using GraphPad Prism 6 software.

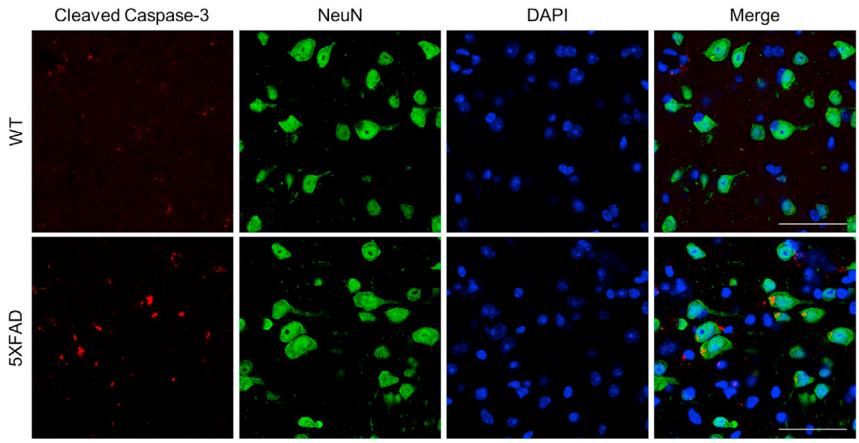

Figure 2. Neuronal apoptosis in aged 5XFAD mice

Representative pictures of apoptotic neurons detected by cleaved caspase-3 and NeuN double staining in the cortex of 9-month-old WT and 5XFAD mice. Scale bar, 50 μm.

⚠ CRITICAL: The species of normal serum used for blocking buffer should be consistent with the species of your secondary antibodies. If you use goat anti-rabbit IgG and goat anti-guinea pig IgG secondary antibodies, you should use goat normal serum for blocking.

Nissl staining for neuronal density analysis

Timing: 4–5 h

Rationale: Cresyl violet acetate solution is able to stain Nissl substance in the cytoplasm of neurons. Nissl granules are purple, nuclei of glial cells and endothelial cells are slightly bluer than Nissl granules. Thus, the neuronal density in brain section can be measured by Nissl staining using Cresyl violet acetate.

19. Wash brain sections with 1× PBS for 3 times, 10 min for each time, to thoroughly remove anti-freezing solution.
20. Mount sections on gelatin-coated slides and allow them to dry at least for 1 h at room temperature.
21. Perform Nissl staining as shown below:

Step	Solution	Time of immersion
1	ddH_2O	10 s
2	0.5% Cresyl violet	10 min
3	ddH_2O	10 s
4	70% ethanol	5 min
5	95% ethanol	1 min
6	95% ethanol containing 0.7% glacial acetic acid	1 min
7	100% ethanol	2 min, twice
8	ethanol: xylene (1: 1)	5 min
9	Xylene	5 min

22. Mount brain sections on slides with neutral balsam.
23. Put cover glass on the slides and push off bubbles and remove extra neutral balsam.
24. Take pictures of brain sections using Olympus BX51 microscope under bright-field with 20X objective lens (Figure 3). For each mouse brain sample, three to five serial sections should be stained, and entire brain region should be pictured for quantification.

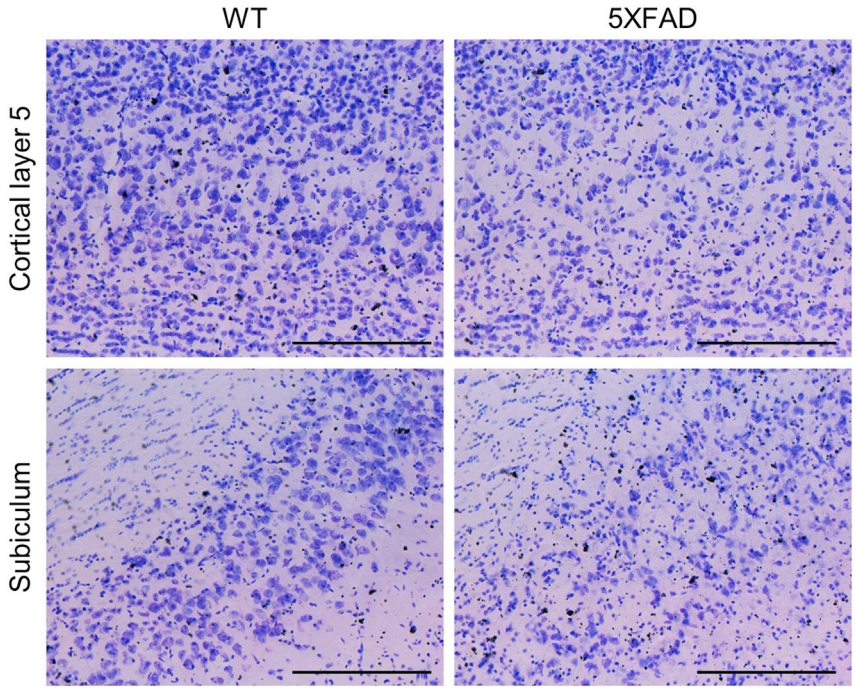

Figure 3. Neuronal loss in cortical layer 5 and subiculum of aged 5XFAD mice

Representative pictures of Nissl staining in the cortex and subiculum of 9-month-old WT and 5XFAD mice. Scale bar, 200 μm.

25. Open pictures with Fiji-ImageJ software, and use modules of "Measure" and "Analyze Particles" to calculate the neuronal density by measuring the brain area and counting the number of total neurons respectively.
26. Statistically analyze the neuronal density in given brain area (cells/mm^2) among several groups using GraphPad Prism 6 software.

⚠ CRITICAL: Aged 5XFAD mice display significant neuronal loss in cortical layer 5 and subiculum of hippocampus according to literature (Oakley et al., 2006). However, significant neuronal loss can also be detected in the dentate gyrus of hippocampus in 9-month-old 5XFAD mice (Zhang et al., 2021). But the neuronal loss in dentate gyrus is hard to be analyzed via Nissl staining method due to compact localization of hippocampal neurons in dentate gyrus. Thus, we recommend to measure neuronal density in dentate gyrus using immunofluorescent staining with anti-NeuN antibody, combining with confocal imaging (Figure 4).

EXPECTED OUTCOMES

Neurodegeneration is a slowly progressive process during the development of Alzheimer's disease in 5XFAD mice. Different experimenters will get different results on the numbers of degenerated neurons, apoptotic neurons or total neurons due to a lot of factors, such as the stage of the mice, the differences between staining reagents as well as the counting methods. However, it will not affect the conclusion on the difference of neuronal apoptosis or neuronal density between different groups, as long as you perform the staining and counting under the same condition and principle.

In our hands, the density of FJC-positive neurons is 30–40 cells/mm^2 in the cortex and hippocampus of 9-month-old 5XFAD mice. However, there is a few FJC-positive neurons, typically less than

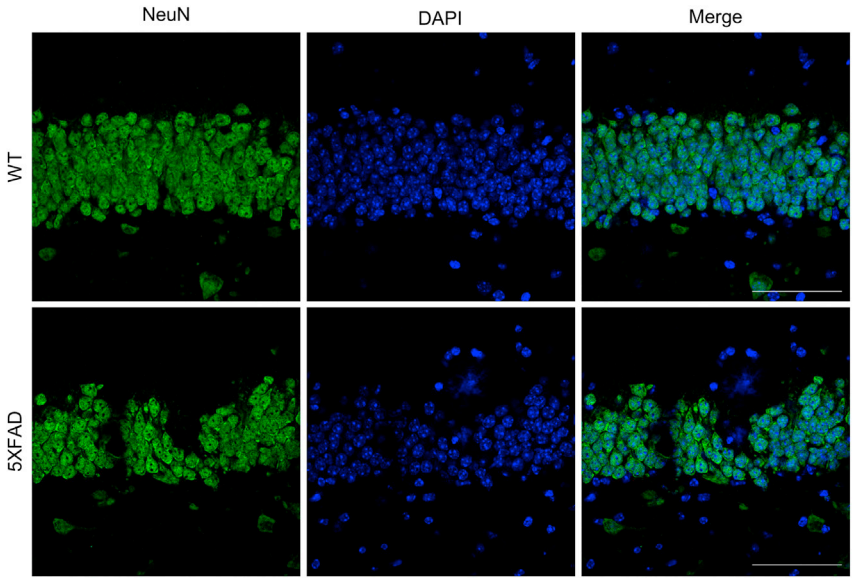

Figure 4. Neuronal loss in dentate gyrus of aged 5XFAD mice

Representative pictures of NeuN immunofluorescent staining in the dentate gyrus of 9-month-old WT and 5XFAD mice. Scale bar, 50 μm.

1 cell/mm^2, in cortex and hippocampus of WT mice, which can serve as a negative control to confirm the reliability of your staining result (Figure 1).

In our experience, the ratio of cleaved caspase-3 positive neurons is 10%–15% in the cortex of 9-month-old 5XFAD mice, versus 2%–4% in 9-month-old WT mice. However, the percentage of cleaved caspase-3 positive neurons in dentate gyrus is relatively low, with a ratio of 1.5%–2% in 9-month-old 5XFAD mice and 0.2%–0.5% in age-matched WT mice.

9-month-old 5XFAD mice exhibit significant neuronal loss in cortical layer 5 and subiculum compared with WT control mice (Figure 3). The neuronal density in cortical layer 5 is about 1,500 cells/mm^2 in WT mice, but it drops to about 1,200 cell/mm^2 in 9-month-old 5XFAD mice. Similarly, the neuronal density in subiculum is about 2,500 cells/mm^2 in WT mice, but it significantly drops to about 1,000 cell/mm^2 in 9-month-old 5XFAD mice.

QUANTIFICATION AND STATISTICAL ANALYSIS

The experimenters should be blinded to genotype when taking pictures for microscopic analysis. For each mouse brain sample, three to five serial sections should be stained for each experiment, and entire brain region should be pictured for quantification. At least four to six fields per section need to be randomly pictured for quantification if the whole-brain imaging cannot be achieved due to the limitation of the microscopes. Comparison between two groups should be analyzed by unpaired two-tailed Student's *t*-test and comparison among over three groups should be analyzed by one- or two-way ANOVA test depending on the treatment on mouse models.

LIMITATIONS

This protocol provides detailed methods to assess neurodegenerative phenotypes in aged 5XFAD mice from neurodegeneration, neuronal apoptosis to final neuronal loss. But this protocol does not evaluate glial markers of neurodegenerative processes (such as GFAP and Iba1 staining) and thus purely focuses on neuronal based analyses. Even though Fluro-Jade C is a specific neurodegenerative marker, the reason why Fluro-Jade C can specifically label degenerated neurons still unclear. In addition, using cleaved caspase-3 antibody to detect neuronal apoptosis remain controversial, as caspase-3 activation

might be involved in synaptic function in the adult brain (Snigdha et al., 2012), thus methods that can detect neuronal apoptotic bodies and/or chromatin condensation, which are more proximal to death by apoptosis, need to be developed. Nissl staining is a widely used method to measure neuronal density. Nissl staining is very easy, cheap and timesaving, but the specificity of neuronal labeling is low as other cell types such as glial cells can also be stained by Cresyl violet, it takes some time to distinguish neurons from other cells when analyzing. By contrast, NeuN (or MAP2) staining is more specific than Nissl staining and contributes to more accurate results. All three staining methods are necessary when analyzing neuronal degeneration and death resulted from neuronal apoptosis, but only Fluro-Jade C and Nissl staining are required when you study other mechanisms of neuronal death.

TROUBLESHOOTING

Problem 1

The brain sections cannot recover to normal shape after FJC staining (step 2d).

Potential solution

Increase the recovering time in 1× PBS and change the 1× PBS for several times.

Problem 2

High green background of FJC staining when imaging using microscope (step 3a).

Potential solution

Wash brain sections with ddH2O for more times and extend the time for washing after FJC staining to remove extra FJC solution completely.

Problem 3

Weak or no signal of cleaved caspase-3 (step 17).

Potential solution

Increase the concentration of anti-cleaved caspase-3 antibody to 1:50.

Problem 4

Over- or under-staining of Nissl (step 21).

Potential solution

Always check the intensity of Nissl staining after submerging in 95% EtOH. If brain sections are over-stained, increase the time of immersion in differentiation solution (95% ethanol containing 0.7% glacial acetic acid) to 1.5–2 min. If brain sections are under-stained, decrease the time of immersion in differentiation solution to 10–30 s or skip differentiation.

Problem 5

There are many small holes in the brain slices when imaging (step 24).

Potential solution

Increase the time of dehydration of mouse brain in 30% sucrose solution. Insufficient dehydration usually causes the formation of ice crystal, which can destroy the normal structure of brain.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Anning Lin (anninglin@nju.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any unique data sets or code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

L.Z. and J.L. optimized and conducted the experiments and wrote the manuscript; A.L. conceived the project and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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