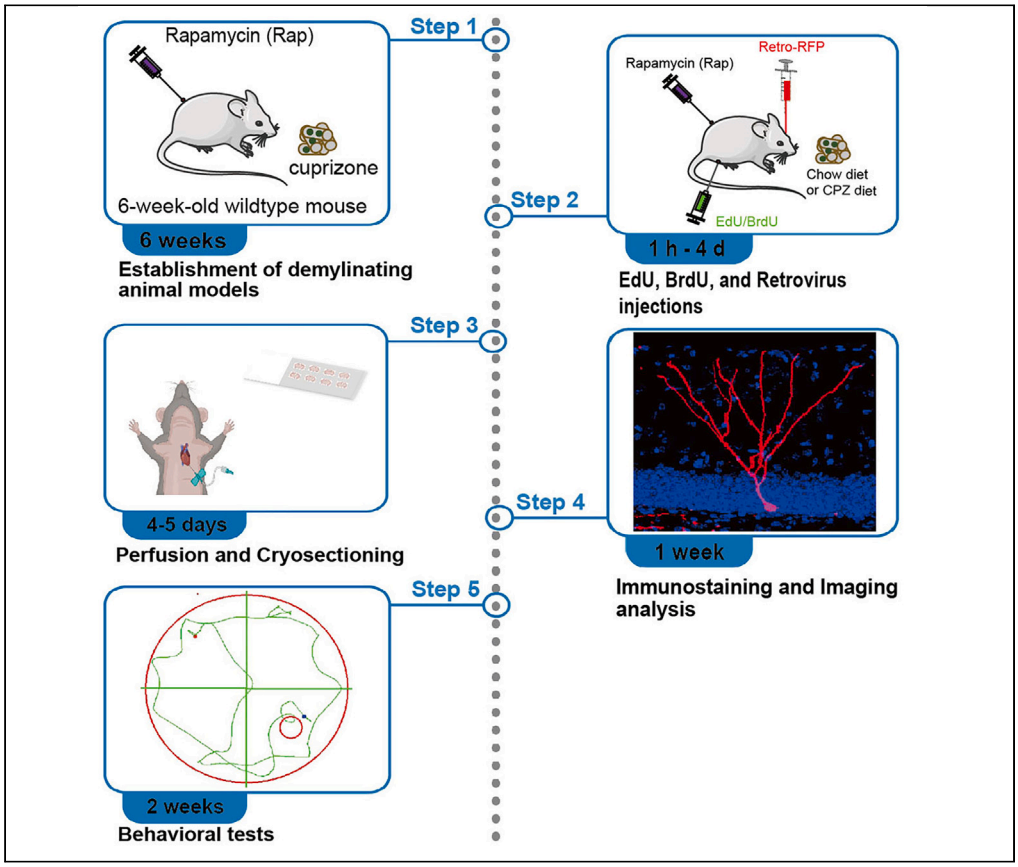
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol to establish a demyelinated animal model to study hippocampal neurogenesis and cognitive function in adult rodents



Cognitive dysfunction is a prevalent feature in multiple sclerosis, a chronic inflammatory demyelinating disease, which may be correlated with the impairment of adult hippocampal neurogenesis. Here, we present a detailed protocol for the induction of cuprizone demyelinated mice to assess the cognitive function and explore the precise mechanisms underlying cognitive deficits in demyelinated hippocampus. We describe steps for behavioral tests, 5-Ethynyl-2′-deoxyuridine (EdU) and bromodeoxyuridine (BrdU) administration, retrovirus packaging and stereotactic injection, hippocampal tissue preparation, and immunofluorescence staining.

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

Yuhan Li,
Changyong Tang,
Yanna Song

tangchy23@mail.sysu.edu.cn (C.T.)
songyn6@mail2.sysu.edu.cn (Y.S.)

Highlights

Protocol for the induction of demyelinating animal models

Step for stereotactic retroviral injection into adult mouse hippocampus

Assessment of hippocampal neurogenesis and neuronal development in demyelinated mice

Li et al., STAR Protocols 5, 103242
September 20, 2024
https://doi.org/10.1016/j.xpro.2024.103242

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol to establish a demyelinated animal model to study hippocampal neurogenesis and cognitive function in adult rodents

Yuhan Li,[1] Changyong Tang,[1,2,3,*] and Yanna Song[1,*]

[1]Department of Neurology, The Third Affiliated Hospital of SUN Yat-sen University, 600 Tianhe Road, Guangzhou 510630, Guangdong Province, China

[2]Technical contact

[3]Lead contact

*Correspondence: tangchy23@mail.sysu.edu.cn (C.T.), songyn6@mail2.sysu.edu.cn (Y.S.)
https://doi.org/10.1016/j.xpro.2024.103242

SUMMARY

Cognitive dysfunction is a prevalent feature in multiple sclerosis, a chronic inflammatory demyelinating disease, which may be correlated with the impairment of adult hippocampal neurogenesis. Here, we present a detailed protocol for the induction of cuprizone demyelinated mice to assess the cognitive function and explore the precise mechanisms underlying cognitive deficits in demyelinated hippocampus. We describe steps for behavioral tests, 5-Ethynyl-2′-deoxyuridine (EdU) and bromodeoxyuridine (BrdU) administration, retrovirus packaging and stereotactic injection, hippocampal tissue preparation, and immunofluorescence staining.

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

BEFORE YOU BEGIN

Cuprizone (CPZ)-induced demyelinated mice model is generally accepted to investigate demyelination in central nervous system (CNS), which is an extensively applicable tool to study the motorial, cognitive, and emotional changes in multiple sclerosis (MS).[2–5] Additionally, to inhibit spontaneous remyelination, CPZ-induced mice are administrated daily intraperitoneal injection with rapamycin (Rap, 10 mg/kg/day).[3,6] Adult hippocampal neurogenesis (AHN) is a continuous process consisting of the proliferation and differentiation of neural stem cells (NSCs) as well as the development of newborn neuron.[7,8] AHN has been reported to be involved in memory formation and emotional regulation in neurological and psychiatric diseases.[7–9] Hence, we aimed to investigate cognitive and neurogenic deficits and explore the link between cognition and AHN in CPZ demyelinated mice. We intraperitoneally administrated EdU and BrdU to mice so as to assess the proliferation and differentiation of NSCs. Additionally, to study neuronal development in CPZ-induced mice, we engineered a red fluorescent protein (RFP)-expressing retroviruses to label newborn neuron and stereotaxically injected them into dentate gyrus (DG) of CPZ/Rap demyelinated hippocampus. In conclusion, CPZ/Rap-induced mice serve as a demyelinating tool for elaborating on the changes of cognition and AHN in demyelinated hippocampus.

Institutional permissions

All experiments are performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and are approved by the Animal Care Committee and the Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University.

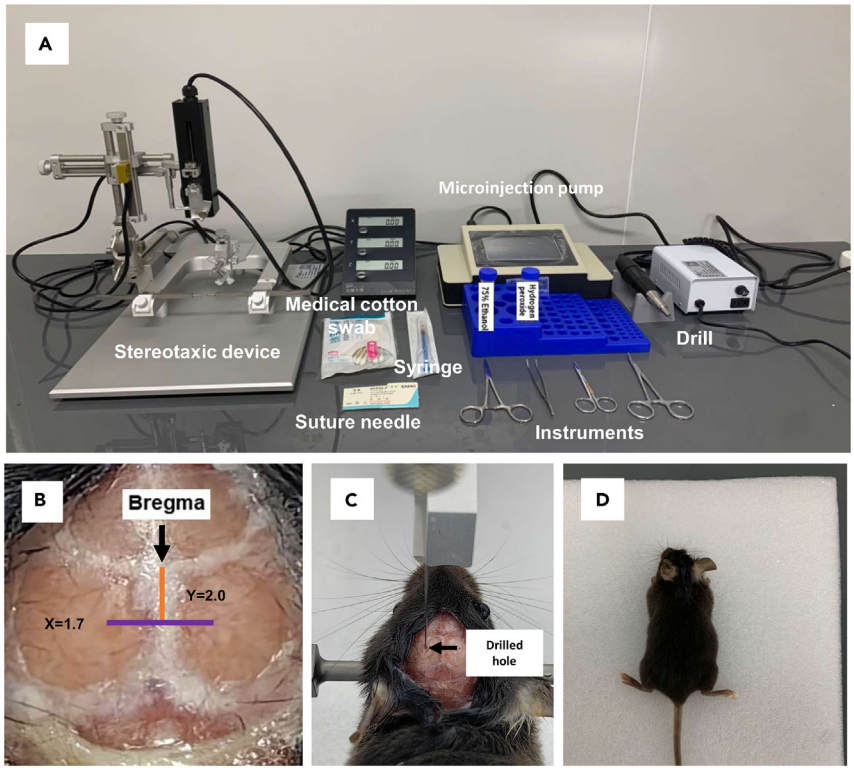


Figure 1. Stereotactic device, surgical instruments and regents, as well as steps associated with retrovirus injection into DG of hippocampus

(A) Tools and equipment for stereotactic surgery.

(B) Expose the mouse's skull and locate the injection site of DG: −2.0 mm posterior to the bregma, ±1.7 mm lateral to the midline, and 1.9 mm below the dura.

(C) Place micro-syringe into the drilled hole according to the above position.

(D) Stitch the wound with suture needle after surgery.

All animal procedures comply with protocols approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University.

Preparation of CPZ diet, rapamycin, and mice

Timing: ~1 week

1. Prepare CPZ diet.
   a. Mix 20 g CPZ with 10 kg standard chow diet to achieve 0.2% CPZ diet.
   b. Seal CPZ diet and store it at 4°C before the start of the experiment.
2. Prepare a rapamycin solution at 0.5 mg/mL concentration.
   a. Dissolve 10 mg rapamycin in 0.1 mL DMSO and 0.9 mL corn oil to obtain stock solution at 10 mg/mL concentration, store at −80°C, and use it within six month.
   b. Dilute rapamycin with Dulbecco's phosphate-buffered saline (DPBS) to achieve a final concentration of 0.5 mg/mL.
   c. Aliquot in 1.5 mL Eppendorf tubes and store at −80°C.
3. Purchase wild-type C57BL/6 male mice (6 weeks) and house them under standardized conditions with free access to food and water.

Note: Rapamycin is firstly dissolved in 10% DMSO and 90% corn oil to acquire stock solution. Once diluted with DPBS, the solution needs to be stored separately to avoid repeated freezing and thawing.

Preparation for EdU or BrdU solution

Timing: 1 h

4. Prepare an EdU or BrdU solution at the concentration of 10 mg/mL.
   a. Dissolve 10 mg EdU or BrdU in 1 mL DPBS (10 mg/mL).
   b. Fill thermostatic water bath with double distilled water (ddH_2O) and heat it to 42°C.
   c. Vortex and heat repeatedly to completely dissolve EdU or BrdU solution.

Note: Prepare fresh solution each time prior to use. Store EdU and BrdU powders in the dark at −20°C for up to 2 years.

Preparation for retrovirus packaging

Timing: ~1 week

5. Amplify and isolate RVs target plasmid containing RFP (CAG-RFP vector, minimum of 150 μg) and the two helper plasmids (pCMV-Gag-Pol, minimum of 100 μg and VSVG, minimum of 80 μg).
6. Prepare transfection reagent Polyethylenimine (PEI, 1 mg/mL) and other medium, including HEK293T cells complete medium (Dulbecco's modified Eagle's medium (DMEM), 10% Fetal Bovine Serum (FBS) and 1% Penicillin Streptomycin (PS)), Opti-MEM I Reduced Serum Media, and DPBS.
7. Culture HEK293T cells in complete DMEM medium in 5 × 15 cm dishes for transfection and incubate cells at 37°C, 5% CO_2.

Note: The recommended confluence of HEK293T cells is 80%.

Preparation of retrovirus stereotactic injection

8. Prepare reagents required for stereotactic injection: 75% ethanol, isoflurane, hydrogen peroxide, ophthalmic ointment, and RVs.
9. Sterilize stereotactic equipment and surgical instruments with 75% ethanol (Figure 1A).
10. Prepare the mice for stereotactic surgery.
    a. Anesthetize 8-week-old male mice with 2% isoflurane inhalation.
    b. Fix anesthetized mice on the stereotaxic frame.

Note: The isoflurane can be replaced by pentobarbital sodium (50 mg/kg, intraperitoneal (i.p.) injection). Check and make sure the mice under the state of deep anesthesia before surgery. Ensure the mouse's head is successfully fixed without any movements during stereotactic surgery.

Preparation of transcardiac perfusion-fixed and immunostaining solutions

Timing: ~2 h

11. Prepare peristaltic pump and surgical tools, including dissecting forceps, ophthalmic forceps, hemostat, tissue scissors, fine scissors, 1 mL syringe and infusion needle.

12. Prepare adequate reagents: Phosphate-buffered saline (PBS), 0.2 M phosphate buffer (PB, stored at 25°C for up to 1 month), 4% paraformaldehyde (PFA, stored at 4°C for up to 2 weeks in the dark), 30% sucrose, cryoprotectant solution (stored at 4°C for up to 1 month), and Polyvinyl alcohol (PVA)-DABCO solution.

Note: See Materials and Equipment section for the components and storage methods of the above regents. It is recommended to be best prepare fresh solution prior the experiment.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rat anti-BrdU (1:1,000)	Abcam	Cat# 6326; RRID: AB_305426
Mouse anti-GFAP (1:1,000)	Cell Signaling Technology	Cat# 3670S; RRID: AB_561049
Rabbit anti-DCX (1:500)	Cell Signaling Technology	Cat# 14802S; RRID: AB_2798619
Rabbit anti-NeuN (1:500)	Abcam	Cat# 177487; RRID: AB_2532109
Rabbit anti-Sox2 (1:500)	Abcam	Cat# 97959; RRID: AB_2341193
Rabbit anti-RFP (1:1,000)	MBL	Cat# PM005; RRID: AB_591279
Rabbit anti-MBP (1:500)	Cell Signaling Technology	Cat# 78896S; RRID: AB_2799920
Rabbit anti-PLP (1:500)	Cell Signaling Technology	Cat #28702
Rabbit anti-Ng2 (1:500)	Sigma-Aldrich	Cat# AB5320; RRID: NM_001897.4
Mouse anti-CC1 (1:100)	Sigma-Aldrich	Cat# OP80; RRID: AB_2057371
Goat anti-rabbit 647 (1:1,000)	Invitrogen	Cat# A-21245, RRID: AB_2535813
Goat anti-mouse 647 (1:1,000)	Invitrogen	Cat# A-21235, RRID: AB_2535804
Goat anti-rat 568 (1:1,000)	Invitrogen	Cat# A-11077, RRID: AB_2534121
Goat anti-rabbit 568 (1:1,000)	Invitrogen	Cat# A-11011, RRID: AB_143157
Goat anti-rabbit 488 (1:1,000)	Invitrogen	Cat# A-11008, RRID: AB_143165
Goat anti-mouse 488 (1:1,000)	Invitrogen	Cat# A-11001, RRID: AB_2534069
Bacterial and virus strains		
DH5a	Tiangen	Cat#CB101-02
Trans110 chemically competent cell	TransGen Biotech	Cat#CD311-02
Retrovirus	This paper	N/A
Critical commercial assays		
EdU cell proliferation kit	Beyotime	Cat#ST067
Chemicals, peptides, and recombinant proteins		
Goat serum	Gibco	Cat#16210072
Opti-MEM I reduced serum media	Gibco	Cat#31985070
Dulbecco's modified Eagle's medium (DMEM) medium	Wisent	Cat# 319-005-CL
Dulbecco's phosphate-buffered saline (DPBS)	Gibco	Cat#14190136
Phosphate-buffered saline (PBS)	Gibco	Cat#C10010500BT
Penicillin/streptomycin	Gibco	Cat#15140-122
Erythromycin eye ointment	Baiyunshan	Cat#212020050
Fetal bovine serum (FBS)	PAN-Seratech	Cat#ST30-3302
Polyethyleneimine (PEI)	Proteintech Group	Cat#PR40001
BrdU	Sigma	Cat#B9285
Isoflurane	RWD	Cat#R510-22-10
Tris-HCL	Sigma	Cat#T3253
PVA-polyvinyl alcohol	Sigma	Cat#p8136
DABCO	Sigma	Cat#D2522
Sodium phosphate monobasic anhydrous (NaH_2PO_4)	Solarbio	Cat#S5141
Sodium phosphate dibasic anhydrous (Na_2HPO_4)	Solarbio	Cat#D9790

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Paraformaldehyde (PFA)	Sigma	Cat#158127
Sodium hydroxide (NaOH)	Sigma	Cat#S8045
Glycerol	Fisher	Cat#G33-1
Ethylene glycol	Sigma	Cat#324558
0.9% normal saline solution	Procell	Cat#PB180353
DAPI for nucleic acid staining	Sigma-Aldrich	Cat# D9542
Triton X-100	Beyotime	Cat# ST79
Experimental models: Cell lines		
HEK293T	ATCC	N/A
Experimental models: Organisms/strains		
Mouse: C57BL/6N, 6-week-old, males	Charles River	Strain code: 213
Recombinant DNA		
CAG-RFP (retroviral vector)	This paper	N/A
Software and algorithms		
LAS X software	Leica Microsystems	N/A
SMART software V3.0	SMART Technologies	N/A
ImageJ	National Institutes of Health	RRID: SCR_003070; https://imagej.net/ij/download.html
GraphPad Prism 9	GraphPad Software	RRID:SCR_002798; https://www.graphpad.com/
Other		
69100 rotational digital stereotaxic instruments for mice and rat	RWD	Cat# K69100
KDS Legato 130 syringe pump	RWD	Cat#KDS LEGATO 130
Drill	RWD	Cat#78001
Rodent anesthesia machine	RWD	Cat#R500
Syringe holder	RWD	Cat#68218
1 mL syringe	KDL	Cat#60017031
1.5 mL microcentrifuge tubes	Axygen	Cat#MCT-500-C-S
15 mL centrifuge tubes	Corning	Cat#CLS430055
50 mL centrifuge tubes	Corning	Cat#430291
75 cm^2 culture flasks	Corning	Cat#430720
Centrifuge tubes	Backman Coulter	Cat#344058
Ultracentrifuge	Backman Coulter	Cat#Optima XE-100 A94516
Vortex mixer	Gilson	Cat# 36110740
Animal platform	RWD	Cat#68607
Delicate scissor	RWD	Cat#S12003
Surgical handle	RWD	Cat#S32001
Delicate forceps	RWD	Cat#F12010
Haemostatic forceps	RWD	Cat#F21011
Needle holder	RWD	Cat#F31031
Heating pad	Nuansandong	Cat#CDT30X35
Suture	RWD	Cat#F35305-50
Suture needle	RWD	Cat#F35401-50
A drill bit	RWD	Cat#78041
Electric razor	BDL	Cat#K9
Microsyringe	Hamilton	Cat#87943
Microsyringe needle	Hamilton	Cat#780305
Cotton swab	Winner	Cat#601-020764-01
150 mm dishes	Corning	Cat#430599
Vacuum filter/storage bottle system	Biosharp	Cat# BS-QT-037
Electronic balance	Yingheng	Cat# YHM-10002Y
Sliding microtome	Thermo Scientific	Cat#HM430
Cuprizone	Sigma-Aldrich	Cat# C9012-25G

MATERIALS AND EQUIPMENT

0.2 M phosphate buffer (PB)

Reagent	Final concentration	Amount
NaH_2PO_4 (MW156.01)	0.2 M	12.168 g
Na_2HPO_4 (MW 358.14)	0.2 M	43.676 g
ddH_2O	N/A	≈1000 mL
Total	0.2 M	1000 mL

Note: Dissolve first with 800 mL ddH_2O and then add ddH_2O until volume reaches 1 L. Adjust pH to 7.4. Pure the solution by filtering with a 0.22 μm filter. The powders should be stored at 25°C. Fresh medium should be prepared or stored at 25°C for up to 1 month.

4% paraformaldehyde (PFA)

Reagent	Final concentration	Amount
PFA	4%	40 g
NaOH	1 M	1 mL
0.2 M PB	0.1 M	500 mL
ddH_2O	N/A	500 mL
Total	N/A	1000 mL

Note: The fresh medium should be stored at 4°C in the dark for up to 2 weeks. Perform all perfusions should use appropriate personal protective equipment, such as nitrile gloves, lab coat.

30% sucrose solution

Reagent	Final concentration	Amount
Sucrose	30%	30 g
0.2 M PB	0.2 M	70 mL
Total	N/A	100 mL

Note: The fresh solution should be stored at 4°C for up to 3 mouths. Dissolve 30 g sucrose in 0.2 M PB solution on a vortex mixer to obtain the final volume of 100 mL.

Cryoprotectant solution

Reagent	Final concentration	Amount
Glycerol	25%	50 mL
Ethylene glycol	25%	50 mL
0.2 M PB	0.1 M	100 mL
Total	N/A	200 mL

Note: Fresh medium should be prepared or stored at 4°C for up to 1 month.

Blocking buffer

Reagent	Final concentration	Amount
Triton X-100	2.5%	250 μL
Goat serum	3%	300 μL
PBS	0.01 M	9.45 mL
Total	N/A	10 mL

Note: Fresh medium should be prepared or stored at 4°C for up to 1 week.

Polyvinyl alcohol (PVA)-DABCO solution

Reagent	Final concentration	Amount
Glycerin	240 mg/mL	6 g
PVA	96 mg/mL	2.4 g

(*Continued on next page*)

Continued

Reagent	Final concentration	Amount
ddH_2O	N/A	6 mL
0.2 M Tris-HCl	0.1 M	12 mL
DABCO	25 mg/mL	0.625 g
Total	N/A	≈25 mL

Note: Add 0.2 M Tris-HCl to adjust the pH to 8–8.5. The solution is recommended to aliquot in 1 mL Eppendorf tubes and store at −20°C to avoid refreezing frequently. The medium should be stored at −20°C for up to 6 months.

STEP-BY-STEP METHOD DETAILS

Establishment of a CPZ/Rap-demyelinating model

Timing: ~6 weeks

Here, we describe the steps for the establishment of CPZ/Rap-demyelinating mouse model.

1. Feed 6-week-old wild-type mice with CPZ diet for 6 weeks.
2. Inject intraperitoneally rapamycin (Rap, 10 mg/kg/day) into CPZ mice every day.
3. House and breed CPZ/Rap mice at 21°C on a 12/12-h light/dark cycle.

Note: Mice were housed in the Guangdong Laboratory Animals Monitoring Institute with free access to enough water and food.

⚠ CRITICAL: To prevent spontaneous remyelination, CPZ-demyelinating mice were given daily intraperitoneal injection of rapamycin.[3,6,10]

Production of retrovirus

Timing: 1–2 weeks

Here, we provide the detailed steps for the retrovirus packaging.

4. Prepare 293T cell to reach 80% confluence in 150 mm dish before transfection.
5. Prepare transfection mixture of the DNA plasmids.
   a. Dilute and mix two helper plasmids and target plasmid in Opti-MEM to obtain a final volume of 5 mL (Solution A).

Vector DNA	150 μg
pCMV-Gag-Pol	100 μg
VSVG	80 μg

   b. Prepare and dilute polyethyleneimine (PEI, 1mg/ml) in Opti-MEM with the ratio of 1 μg DNA to 2.5 μL PEI to obtain a final volume of 5 mL (Solution B).
   c. Mix solution A and solution B and incubate for 20 min at 25°C.
6. Add 2 mL DNA plasmids-PEI complex into 293T cell per dish and incubate cells at 37°C, 5% CO_2.
7. Replace fresh DMEM complete medium after 5 h.
8. Collect medium supernatant containing retroviral viral particles 48, 72, and 96 h after transfection.
9. Concentrate supernatant at 4,000 g for 15 min and filter through a 0.22 μm filter to remove cellular debris.
10. Harvest viral particles by ultracentrifugation for 2 h at 49000 g at 4°C.

11. Resuspend viral particles with 100 μL DPBS and then aliquot and store the virus at −80°C until further use.

Note: Lipofectamine 2000 is also an alternative transfection reagent apart from PEI. Use centrifuge model (SW 32Ti) and rotor (SW 32 Ti rotor with part number of 326823). For more detailed protocol of virus packaging, please refer to Li Xu et al.[11]

⚠ CRITICAL: For cell survival and optimal transfection efficiency, the exposure time of DNA plasmids-PEI transfection complex should not exceed 6 h. It is recommended to confirm transfection efficiency 48 h after transfection. Please refer to Li Xu et al. for details on verification of transfection efficiency.[11] For more detailed protocol of virus titer determination, please see Li Xu et al.[11]

Stereotactic injection of retrovirus

Timing: 1 h per mouse

Inject RFP-expressing retroviruses into DG of hippocampus to scrutinize newborn neuronal development.

12. Fixate the head of anesthetized mouse on a stereotactic frame.
13. Expose the skull of anesthetized mouse.
    a. Shave the head of mouse with pet shaver.
    b. Cut the skin of mouse head with tissue scissors.
    c. Wipe the skull with sterile cotton swab to expose bregma and lambda.
14. Mark the position of the hippocampus coordinating relative to bregma: AP: 2.0 mm, ML: 1.7 mm, DV: 1.9 mm (Figure 1B).
15. Drill a small hole in the surface of the skull after positioning.
16. Inject 1 μL retrovirus into the above stereotaxic coordinate of hippocampus at a rate of 0.5 μL/min (Figure 1C).
17. Slowly remove the microinjection syringe 5 min after infusion to allow complete diffusion of RVs.
18. Repeat steps 14–17 for another hippocampus.
19. Suture the wound with absorbable thread after injection (Figure 1D).
20. Clean the mouse head with sterile cotton ball to avoid infection.
21. Place the mouse on a heating pad for quick recovery of consciousness.
22. Return the mice to their home cages.

Note: Prepare virus on ice before the stereotaxic surgery. It is advisable to smear ophthalmic ointment to protect eyes of mice from bright light burns. After each injection, microinjection syringe should be cleaned with sterile ddH_2O.

⚠ CRITICAL: To better expose the skull, operator can also wipe the skull with sterile cotton swab dipped in hydrogen peroxide. Steps to deliver virus into hippocampus should be performed meticulously as mentioned above for prevent further damage. For more detailed protocol of stereotactic injection see.[12,13]

BrdU injection

Timing: 4 days

Two weeks after CPZ/Rap treatment, mice were injected intraperitoneally with BrdU to analyze NSCs differentiation.

23. Administer mice with BrdU at a daily dose of 50 mg/kg i.p. for 4 consecutive days.
24. Collect the brains of mice 2 and 4 weeks after BrdU injection to calculate the count of differentiated NSCs *in vivo*.

EdU injection

Timing: 1 h

Six weeks after CPZ/Rap administration, mice receive EdU injection to assess NSCs proliferation.

25. Treat mice with EdU at 100 mg/kg i.p. and then sacrificed 2 h after injection to assess the proliferation of NSCs *in vivo*.

Note: Use a different cohort of mice for EdU injection.

Intracardiac perfusion and brain slicing

Timing: 4–5 days

Perfuse the mice via surgery and obtain brain slices by using a frozen microtome.

26. Anesthetize mice with 2% pentobarbital sodium via intraperitoneal injection.

Note: Make sure mice are unresponsive and keep a state of deep anesthesia via toe pinch-response before surgery.

⚠ CRITICAL: It is crucial to test the depth of anesthesia before surgery and maintain the deep anesthesia during intracardiac perfusion.

27. Expose the heart of mice.
    a. Firmly pin the mouse's four paws to a Styrofoam by using needles.
    b. Open the chest with tissue scissors to expose the heart.

Note: Don't hurt the heart and other organs during the perfusion.

28. Make a small incision on the right atrial appendage with fine scissors.
29. Secure the heart with ophthalmic forceps and insert the needle into the left ventricle of mice.

Note: Carefully adjust the needle angle and gently advance the needle into left ventricle to avoid hurting the left atrium or right ventricle.

30. Perfuse the mice with 30 mL of 1× PBS at a flow rate of 10 mL/min.

Note: During the process of intracardiac perfusion with PBS, the liver gradually turns from red to pale. If the needle tip inserts into right ventricle, pulmonary edema will occur.

⚠ CRITICAL: Removal of all air bubbles in the tubing is essential for successful perfusion. The key to determining the success of intracardiac perfusion is to observe the color change of the liver.

31. Steadily perfuse 30 mL of 4% ice-cold paraformaldehyde (PFA) at the same rate.

Note: As PFA is perfused into circulation, mice exhibit signs of opisthotonos, characterized by muscle contractions, neck stiffness, and limbs extension.

⚠ CRITICAL: The solution of PFA is best prepared fresh and placed on ice during perfusion.

32. Gently detach the brain with ophthalmic forceps, tissue scissors, and fine scissors.
33. Place the mouse brain in 4% PFA for 24 h at 4°C for further fixation.
34. Transfer the brain into 30% sucrose solution at 4°C until fully submerged.

⚠ CRITICAL: The brain dehydrates and shrinks in sucrose solution and settles to the bottom of the tube.

35. Slice brain into 40 or 80 μm thickness via a frozen microtome across the entire anterior-posterior extent of the hippocampus using a frozen microtome. Frozen brain blocks were cut into 40 or 80 μm thick sections using a frozen microtome.

Note: Collect brain sections across the entire anterior-posterior extent of the hippocampus. Cut brain into 40-μm-thick coronal sections for immunofluorescence staining. Slice retrovirus-injected brain into 80 μm thickness for neuronal complexity analysis.

36. Store brain sections in cryoprotectant solution at 4°C.

Immunofluorescent staining for myelination

Timing: 2 days

To verify the negative effect of CPZ/Rap administration on myelination, we assess the density of myelin proteins labeled with myelin basic protein (MBP) and proteolipid protein (PLP), as well as the number of oligodendrocytes including $Ng2^+$ oligodendrocyte precursor cells (OPCs) and $CC1^+$ mature oligodendrocytes.

37. Select 40-μm-thick brain sections from cryoprotectant solution.
38. Wash the brain sections three times for 5 min each in 1× PBS at 25°C, accomplished by gently shaking.
39. Transfer brain sections to blocking buffer for 1 h at 25°C to reduce nonspecific binding.
40. Incubate sections separately with Rabbit anti-MBP, Rabbit anti-PLP, Rabbit anti-Ng2, and Mouse anti-CC1 (1:500) at 4°C for 24 h.
41. Repeat the step 38.
42. Incubate brain slices with diluted secondary antibodies (1:1,000) including Goat anti-rabbit 568, Goat anti-rabbit 488, Goat anti-rabbit 647, and Goat anti-mouse 647 for 2 h at 25°C in the dark.
43. Wash the brain slices with 0.01 M PBS three times.
44. Counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for 30 min at 25°C in the dark.
45. Wash the brain slices with PBS (0.01 M).
46. Mount slices with PVA-DABCO solution and seal with coverslip.
47. Store sections at 4°C in the dark before imaging.

Note: Observe the density of myelin proteins and the count of oligodendrocytes to determine whether CPZ/Rap- demyelinated mice are successfully established.

BrdU detection and immunofluorescent staining

Timing: 2 days

BrdU detection is used to examine the differentiation of NSCs.

48. Repeat the steps 37–38.
49. Incubate sections in 2 M HCl for 30 min at 37°C using thermostatic water bath.

Note: Dilute concentrated hydrochloric acid to 2 M with ddH_2O and store at 25°C.

50. Incubate sections in 0.1 M Borate buffer for 30 min at 25°C.

Note: Dilute borate solution with ddH_2O.

51. Wash the brain sections with PBS three times, each time for 5 min at 25°C.
52. Transfer slices to blocking buffer for 1 h at 25°C.
53. Incubate sections with primary antibodies.
    a. Dilute primary antibodies such as Rat anti-BrdU, Rabbit anti-DCX, and Rabbit anti-NeuN in blocking buffer (1:500).
    b. Incubate sections with primary BrdU/DCX or BrdU/NeuN antibody in shaker (10–20 g) at 4°C for 24 h.
54. Repeat the step 51.
55. Incubate sections with diluted secondary antibodies (1:1,000) including Goat anti-rat 568 and Goat anti-rabbit 488 for 2 h at 25°C in the dark.
56. Repeat the steps 43–47.

Immunofluorescent staining and EdU detection

Timing: 2 days

EdU immunostaining is used to assess the proliferation of NSCs.

57. Select 40-μm-thick brain slices from cryoprotectant solution.
58. Wash the brain slices three times for 5 min each in 1× PBS at 25°C.
59. Incubate the brain sections in 0.5% Triton X-100 for 30 min at 25°C.
60. Repeat the step 58.
61. Prepare the Click Reaction Solution according to the manufacturer's instructions (https://www.beyotime.com/product/ST067-50mg.htm).

Note: It is essential to prepare the Click Reaction Solution in strict accordance with the order and volume of the components mentioned above, otherwise the reaction may not be effective.

⚠ CRITICAL: Prepare Click Reaction Solution fresh and use it immediately.

62. Incubate the slices with appropriate Click Reaction Solution at 25°C for 30 min in the dark.
63. Repeat the step 58 away from light.
64. Transfer brain sections to blocking buffer for 1 h at 25°C.
65. Incubate the brain sections with primary antibodies including Mouse anti-GFAP and Rabbit anti-Sox2 (1:500) away from light.
66. Repeat the step 58 in dark.
67. Incubate slices with Goat anti-mouse 647 and Goat anti-rabbit 568 for 2 h at 25°C and protect them from light.
68. Repeat the steps 43–47 in dark.

Note: Conduct steps 61–68 in dark to prevent fluorescence loss during the above experiment.

Examination of newborn neuron development

Timing: 2 days

Harvest the mice at 4 weeks after retrovirus injection. Cut the brain into 80 μm and incubate slices with RFP related antibodies to obtain the image of neurons infected with retroviruses. Analyze the dendritic length and complexity of RFP^+ newborn neurons using Sholl analyses. Capture the image with a 60× per objective lenses on a laser confocal microscope for dendritic spine density analyses.

69. Select 80-μm-thick brain sections labeled with RFP from cryoprotectant solution under a fluorescent microscope.
70. Wash the brain sections three times for 5 min each in 1× PBS at 25°C.
71. Transfer brain sections to blocking buffer for 1 h at 25°C.
72. Remove the blocking buffer and add primary antibody of Rabbit anti-RFP (1:500).
73. Repeat the step 70.
74. Incubate slices with Goat anti-rabbit 568 for 2 h at 25°C in dark.
75. Repeat the steps 43-47 in dark.

Note: Analyze at least 30 cells per group from at least 4 different animals for dendritic morphology and spine density analyses.

Behavioral assessment

Timing: ~2 weeks

This process involves the steps for behavioral tests to assess cognitive function of mice.

Behavioral tests including the Morris Water Maze (MWM) and the Reverse Morris Water Maze (RMWM) are conducted to evaluate cognitive function of mice after 6 weeks of CPZ/Rap administration. Traveling pathway, latency to reach the platform, and the number of platform crossings were recorded to measure spatial memory ability of mice.

Note: RMWM test was performed 48 h after the MWM test to eliminate work memory. For more detailed protocol of MWM and RMWM tests see.[14–16]

EXPECTED OUTCOMES

To explore the effect of demyelination on hippocampal neurogenesis and cognition, we established a CPZ/Rap demyelinated model in wild-type mice by feeding with CPZ diet for 6 weeks, accomplished by rapamycin injection. Six weeks after CPZ/Rap treatment, we investigated the proliferation and differentiation of NSCs by counting the number of EdU and BrdU cells (Figures 2A and 2B). Additionally, we stereotaxically injected retrovirus to label newborn neuron and analyzed the neuronal development (Figures 3A and 3B). We also sought to elucidate the role of CPZ/Rap administration in cognitive and affective function. Six weeks after CPZ/Rap treatment, CPZ/Rap demyelinated mice were successfully established with significant myelin loss, exhibiting substantial reduction in MBP and PLP and decreased count of Ng2 and CC1 positive oligodendrocytes (Figure 2C). As anticipated, CPZ/Rap administration impeded NSCs proliferation, resulting in decreased EdU^+ cells, $EdU^+GFAP^+Sox2^+$ radial glia-like (RGL) NSCs cells and transiently amplifying progenitor (TAP) $EdU^+GFAP^-Sox2^+$ cells in the DG area of hippocampus (Figures 2D and 2E). Subsequently, CPZ/Rap inhibited NSCs differentiation, manifesting with reduced immature neurons ($BrdU^+DCX^+$) and mature neurons ($BrdU^+NeuN^+$) (Figures 2F and 2G). Additionally, CPZ/Rap significantly impaired neuronal dendritic length and complexity (Figure 3C), as well as spine density (Figure 3D). Increased escape latencies and diminished platform crossings in MWM and RMWM were observed in CPZ/Rap

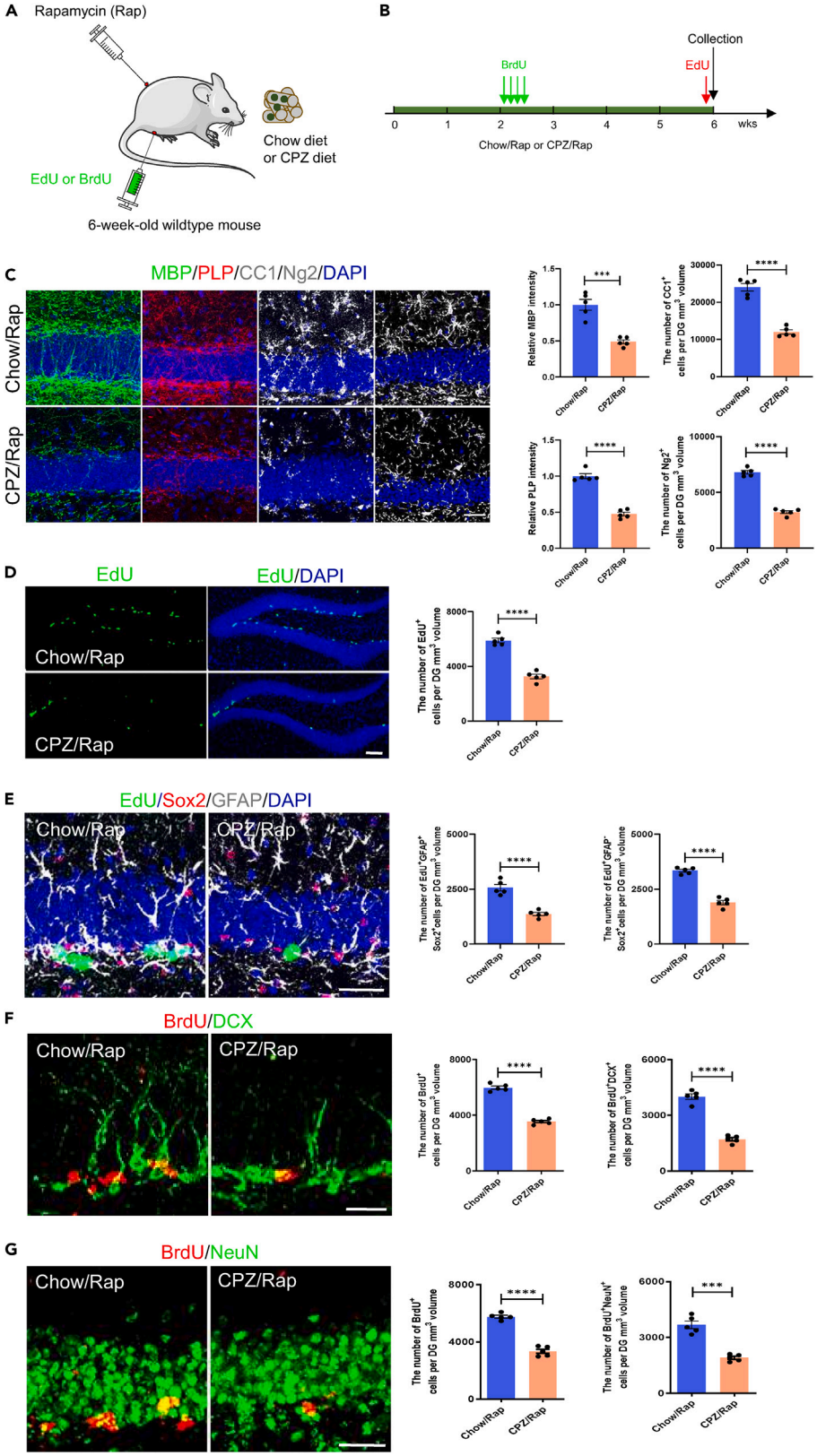

A
Rapamycin (Rap)
Chow diet
or CPZ diet
EdU or BrdU
6-week-old wildtype mouse
B
Collection
BrdU
EdU
0
1
2
3
4
5
6
wks
Chow/Rap or CPZ/Rap
C
MBP/PLP/CC1/Ng2/DAPI
Chow/Rap
CPZ/Rap
Relative MBP intensity
The number of CC1+ cells per DG mm3 volume
Relative PLP intensity
The number of Ng2+ cells per DG mm3 volume
D
EdU
EdU/DAPI
Chow/Rap
CPZ/Rap
The number of EdU+ cells per DG mm3 volume
E
EdU/Sox2/GFAP/DAPI
Chow/Rap
CPZ/Rap
The number of EdU+GFAP+ Sox2+ cells per DG mm3 volume
F
BrdU/DCX
Chow/Rap
CPZ/Rap
The number of BrdU+ cells per DG mm3 volume
The number of BrdU+DCX+ cells per DG mm3 volume
G
BrdU/NeuN
Chow/Rap
CPZ/Rap
The number of BrdU+ cells per DG mm3 volume
The number of BrdU+NeuN+ cells per DG mm3 volume

Figure 2. CPZ/Rap treatments impede NSC proliferation and differentiation

(A and B) Schematic diagram and the timeline of the experiments to assay NSC proliferation and differentiation in 6-week-old wild-type mice receiving the Chow/Rap or CPZ/Rap treatment.

(C) Images and quantification of MBP, PLP, CC1, and Ng2 in the DG of Chow/Rap and CPZ/Rap mice. $n = 5$. Scale bars, 50 μm.

(D) Representative images and quantification of total proliferating cells (EdU^+) in the DG of the indicated groups. $n = 5$ animals per group. Scale bar, 100 μm.

(E) Representative images and quantification of $EdU^+GFAP^+Sox2^+$ triple-labelling radial glia-like (RGL) NSCs cells and transiently amplifying progenitor (TAP) $EdU^+GFAP^-Sox2^+$ cells in the DG of the indicated groups. $n = 5$ animals per group. Scale bar, 50 μm.

(F) Representative images and quantification of $BrdU^+$ cells and $BrdU^+$ DCX^+ immature neurons in the DG of the indicated group. $n = 5$ animals per group. Scale bars, 50 μm.

(G) Representative images and quantification of $BrdU^+$ cells and $BrdU^+$ $NeuN^+$ mature newborn neurons in the DG of the indicated groups. $n = 5$ animals per group. Scale bars, 50 μm. All data were presented as the mean ± SEM. Statistical significance was evaluated with Student's t-test. Nonsignificant comparisons were not identified. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

mice, suggesting cognitive impairments in CPZ/Rap mice (Figures 4A–4F). Taken together, CPZ/Rap treatment impaired myelination, hippocampal neurogenesis, memory, and mood.

LIMITATIONS

The CPZ/Rap model, a toxic demyelinating experimental model, is frequently used to study processes related to demyelination and remyelination.[2,3] Unfortunately, mechanisms involving autoimmune and inflammatory events are not suitable for study with CPZ/Rap demyelinating model. Demyelination is observed after about 6 weeks of CPZ/Rap exposure.[17] Thus, the establishment of demyelinating mice is a relatively long process that requires continuous CPZ/Rap exposure. Additionally, gender and weight differences need to be considered, which can influence the extent of the CPZ/Rap-induced demyelinating changes.[17,18] Aged mice appear to require a higher dose of CPZ (0.4% instead of 0.2%) due to the less susceptible to CPZ and higher body weights.[17] Behavioral tests in the CPZ/Rap mice are limited and focus mainly on motor function, memory, learning, and affective state.[4] However, changes in pain, fatigue, vision, and sleep cannot be well investigated and characterized in the CPZ/Rap model.

TROUBLESHOOTING

Problem 1

Fail to induce demyelinating animal model (steps 1–3).

Potential solution

Feed mice with CPZ diet for consecutive 4–6 weeks is the key to establish the CPZ-demyelinated mice. Importantly, to avoid spontaneous remyelination, mice are daily injected with rapamycin (10 mg/kg i.p.).

Problem 2

Unsuccessful retrovirus packaging (steps 4–11).

Potential solution

Ensure 293 T cells reach 80–90% confluence before transfection.

Timely evaluate the efficiency of transfection 48 h after exposure to DNA plasmids-PEI transfection complex.

Limit use no more 100 μL DPBS to resuspend viral particles after virus concentration to obtain higher virus titers.

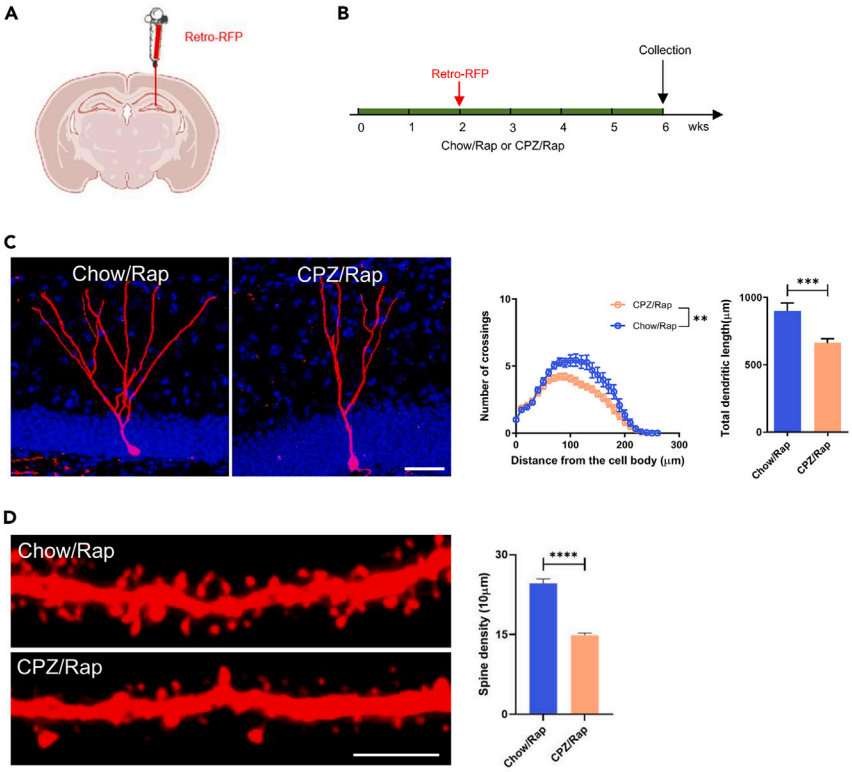


Figure 3. CPZ/Rap administration impairs the development of hippocampal newborn neuron

(A) Schematic diagram of the retroviral vector and stereotaxic grafting of the retrovirus (Retro-RFP) into the DG of mice.

(B) Timeline of the experiments for virus (Retro-RFP) injection in Chow/Rap or CPZ/Rap mice.

(C) Representative images and quantification of the dendritic length and dendritic complexity of RFP^+ newborn neurons of Chow/Rap or CPZ/Rap treated mice. $n = 30$ neurons per group from 5 mice. Scale bar, 50 μm.

(D) Representative images and quantification of the density of dendritic spines of the indicated groups. $n = 30$ neurons per group from 5 mice. Scale bars, 5 μm.

Data were presented as the mean ± SEM. Statistical significance was evaluated with Student's t test; for (C), dendritic complexity was evaluated with two-way ANOVA test. Nonsignificant comparisons were not identified. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Problem 3

Positional deviation during stereotactic injection (steps 12–22).

Potential solution

Fix the head of mouse parallel to the surface of stereotaxic platform without any movements during stereotactic surgery.

Fully expose bregma and lambda to accurately locate the injection site of DG.

Problem 4

Present non-specific staining of brain slides (steps 37–40).

Potential solution

Reduce the concentration of the primary antibody.

Alter another blocking serums.

Replace an alternative primary antibody.

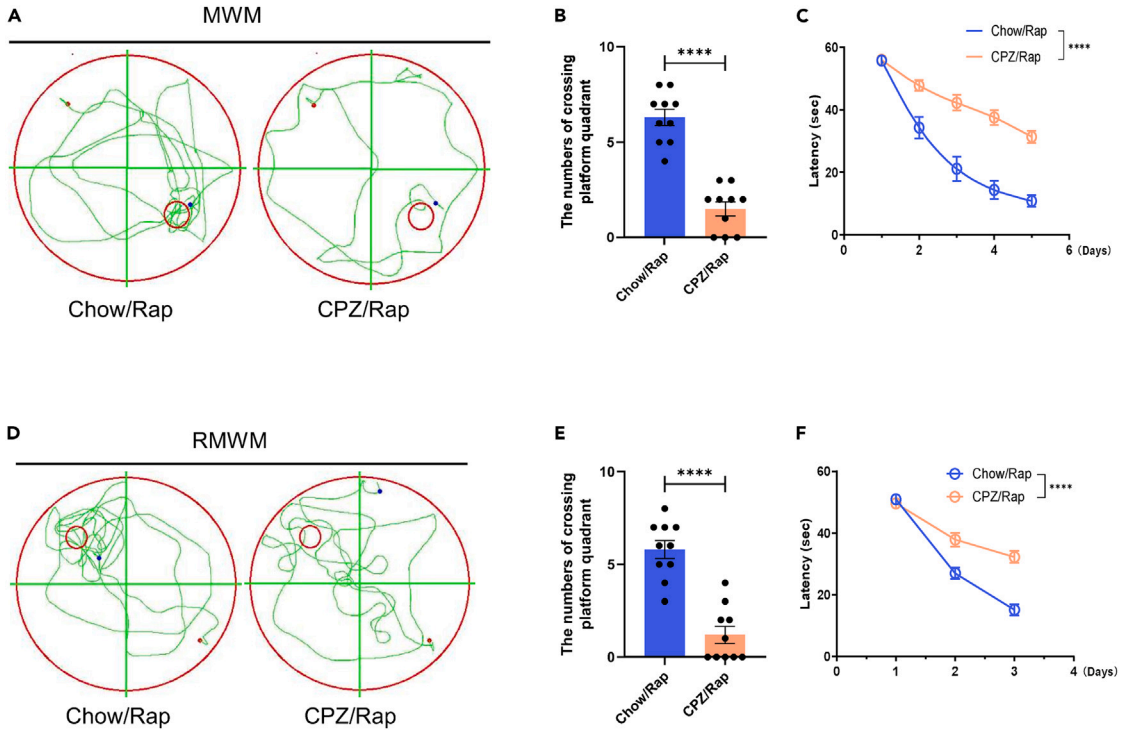


Figure 4. CPZ/Rap mice exhibit impaired spatial memory and cognitive function

(A) Representative MWM movement paths of the indicated groups.

(B) Quantification of the platform crossing numbers in the testing session of MWM. n = 10 animals per group.

(C) Quantification of MWM escape latencies to find the platform during the training sessions. n = 10 animals per group.

(D) Representative RMWM movement paths of the indicated groups.

(E) Quantification of the platform crossing numbers in the RMWM task. n = 10 animals per group.

(F) Quantification of RMWM escape latencies to find the platform during the training sessions. n = 10 animals per group.

Data were presented as the mean ± SEM; for (B) and (E), statistical significance was evaluated with Student's t-test for two-group comparisons; for (C) and (F), significance was evaluated with two-way ANOVA and Tukey's multiple-comparisons test. Nonsignificant comparisons were not identified. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Problem 5

Yield air bubbles during the process of sealing slices (step 46).

Potential solution

The component of PVA-DABCO solution contains glycerin, which can generate bubbles. Hence, it is necessary to centrifuge PVA-DABCO solution to remove air bubbles from the liquid before sealing sections. Coat the glass slide slowly and evenly with cover glass from one side of the slides with tweezers to prevent the formation of air bubbles.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Changyong Tang (tangchy23@mail.sysu.edu.cn).

Technical contact

Further information and requests for the protocol should be directed to and will be fulfilled by the technical contact, Changyong Tang (tangchy23@mail.sysu.edu.cn).

Materials availability

Retrovirus used in this protocol are available under request to the lead contact, Changyong Tang.

Data and code availability

This study did not generate new unique data or code.

ACKNOWLEDGMENTS

We thank Wei Jiang for technical assistance. This research was supported by grants from the National Natural Science Foundation of China (32100787) and the Science and Technology Plan Project of Guangzhou City (202201020489 and 2023A04J1089).

AUTHOR CONTRIBUTIONS

Conceptualization, C.T.; methodology, Y.L. and Y.S.; investigation, Y.L. and Y.S.; writing, Y.L. and Y.S.; funding acquisition, C.T.; supervision, C.T. and Y.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Song, Y., Jiang, W., Afridi, S.K., Wang, T., Zhu, F., Xu, H., Nazir, F.H., Liu, C., Wang, Y., Long, Y., et al. (2024). Astrocyte-derived CHI3L1 signaling impairs neurogenesis and cognition in the demyelinated hippocampus. Cell Rep. *43*, 114226. https://doi.org/10.1016/j.celrep.2024.114226.

2. Matsushima, G.K., and Morell, P. (2001). The neurotoxicant, cuprizone, as a model to study demyelination and remyelination in the central nervous system. Brain Pathol. *11*, 107–116. https://doi.org/10.1111/j.1750-3639.2001.tb00385.x.

3. Kipp, M., Clarner, T., Dang, J., Copray, S., and Beyer, C. (2009). The cuprizone animal model: new insights into an old story. Acta Neuropathol. *118*, 723–736. https://doi.org/10.1007/s00401-009-0591-3.

4. Sen, M.K., Mahns, D.A., Coorssen, J.R., and Shortland, P.J. (2019). Behavioural phenotypes in the cuprizone model of central nervous system demyelination. Neurosci. Biobehav. Rev. *107*, 23–46. https://doi.org/10.1016/j.neubiorev.2019.08.008.

5. Dedoni, S., Scherma, M., Camoglio, C., Siddi, C., Dazzi, L., Puliga, R., Frau, J., Cocco, E., and Fadda, P. (2023). An overall view of the most common experimental models for multiple sclerosis. Neurobiol. Dis. *184*, 106230. https://doi.org/10.1016/j.nbd.2023.106230.

6. Sachs, H.H., Bercury, K.K., Popescu, D.C., Narayanan, S.P., and Macklin, W.B. (2014). A new model of cuprizone-mediated demyelination/remyelination. ASN Neuro *6*, 1759091414551955. https://doi.org/10.1177/1759091414551955.

7. Rocca, M.A., Barkhof, F., De Luca, J., Frisén, J., Geurts, J.J.G., Hulst, H.E., Sastre-Garriga, J., and Filippi, M.; MAGNIMS Study Group (2018). The hippocampus in multiple sclerosis. Lancet Neurol. *17*, 918–926. https://doi.org/10.1016/s1474-4422(18)30309-0.

8. Ming, G.L., and Song, H. (2011). Adult neurogenesis in the mammalian brain: significant answers and significant questions. Neuron *70*, 687–702. https://doi.org/10.1016/j.neuron.2011.05.001.

9. Anacker, C., and Hen, R. (2017). Adult hippocampal neurogenesis and cognitive flexibility - linking memory and mood. Nat. Rev. Neurosci. *18*, 335–346. https://doi.org/10.1038/nrn.2017.45.

10. Zhang, H., Kim, Y., Ro, E.J., Ho, C., Lee, D., Trapp, B.D., and Suh, H. (2020). Hippocampal Neurogenesis and Neural Circuit Formation in a Cuprizone-Induced Multiple Sclerosis Mouse Model. J. Neurosci. *40*, 447–458. https://doi.org/10.1523/JNEUROSCI.0866-19.2019.

11. Xu, L., and Tang, C. (2023). Protocol for lentivirus-mediated delivery of genes to study neurogenesis and cognitive function in adult rodents. STAR Protoc. *4*, 102761. https://doi.org/10.1016/j.xpro.2023.102761.

12. Tang, C., and Guo, W. (2021). Implantation of a mini-osmotic pump plus stereotactical injection of retrovirus to study newborn neuron development in adult mouse hippocampus. STAR Protoc. *2*, 100374. https://doi.org/10.1016/j.xpro.2021.100374.

13. Bosch-Queralt, M., Tiwari, V., Damkou, A., Vaculčiaková, L., Alexopoulos, I., and Simons, M. (2022). A fluorescence microscopy-based protocol for volumetric measurement of lysolecithin lesion-associated de- and re-myelination in mouse brain. STAR Protoc. *3*, 101141. https://doi.org/10.1016/j.xpro.2022.101141.

14. Vorhees, C.V., and Williams, M.T. (2006). Morris water maze: procedures for assessing spatial and related forms of learning and memory. Nat. Protoc. *1*, 848–858. https://doi.org/10.1038/nprot.2006.116.

15. Chen, J.F., Liu, K., Hu, B., Li, R.R., Xin, W., Chen, H., Wang, F., Chen, L., Li, R.X., Ren, S.Y., et al. (2021). Enhancing myelin renewal reverses cognitive dysfunction in a murine model of Alzheimer's disease. Neuron *109*, 2292–2307.e5. https://doi.org/10.1016/j.neuron.2021.05.012.

16. Frame, A.K., Lone, A., Harris, R.A., and Cumming, R.C. (2019). Simple Protocol for Distinguishing Drug-induced Effects on Spatial Memory Acquisition, Consolidation and Retrieval in Mice Using the Morris Water Maze. Bio. Protoc. *9*, e3376. https://doi.org/10.21769/BioProtoc.3376.

17. Norkute, A., Hieble, A., Braun, A., Johann, S., Clarner, T., Baumgartner, W., Beyer, C., and Kipp, M. (2009). Cuprizone treatment induces demyelination and astrocytosis in the mouse hippocampus. J. Neurosci. Res. *87*, 1343–1355. https://doi.org/10.1002/jnr.21946.

18. Irvine, K.A., and Blakemore, W.F. (2006). Age increases axon loss associated with primary demyelination in cuprizone-induced demyelination in C57BL/6 mice. J. Neuroimmunol. *175*, 69–76. https://doi.org/10.1016/j.jneuroim.2006.03.002.