Title

Protocol for culturing neurospheres from progenitor cells in the dentate gyrus of aged mouse hippocampus

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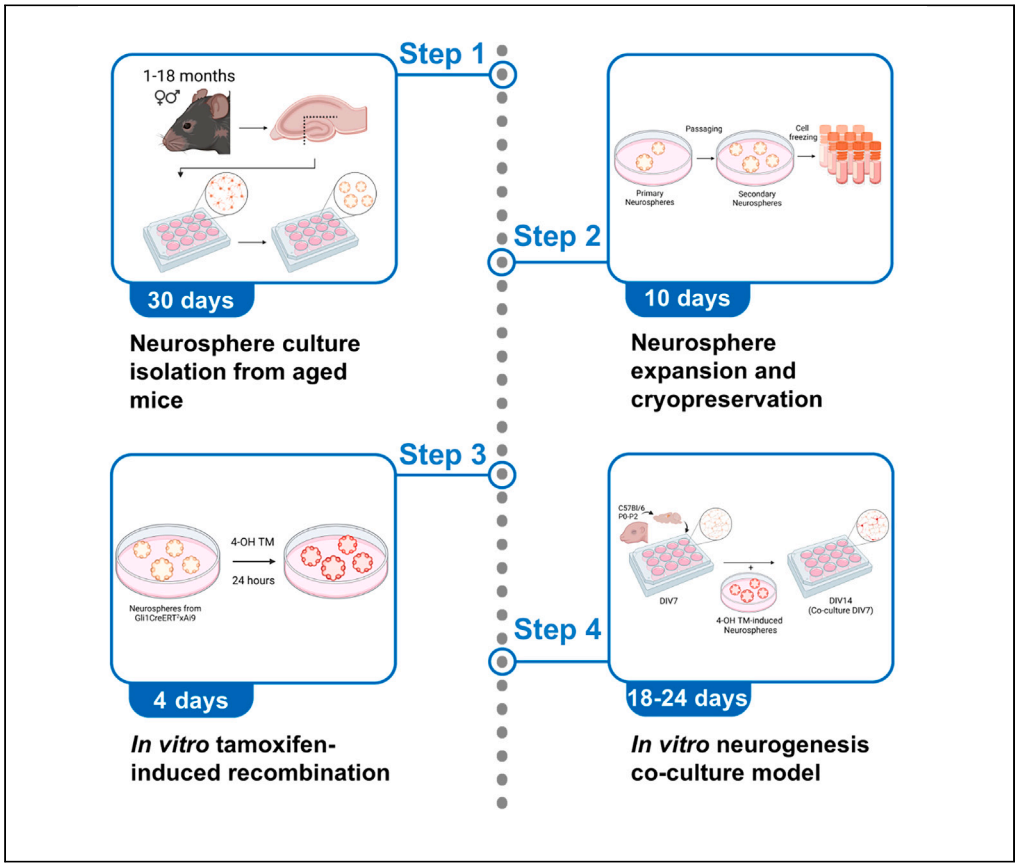

The neurosphere assay is the gold standard for assessing the proliferative and differentiation capacities of neural progenitor cells (NPCs). Here, we present a protocol for isolating, propagating, and maintaining hippocampal neurospheres from adult and aged mice and differentiating cultured NPCs into neurons and astrocytes. We describe steps for establishing a heterochronic co-culture of neurosphere-derived cells with primary neurons. Using neurospheres from old animals enables investigation of the effects of aging on the development and differentiation of newborn neurons.

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

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Highlights

Establishment of neurosphere culture from aged hippocampal dentate gyrus

Expansion of neural precursor cells from the aged brain to neurospheres

Immunofluorescent analysis of molecular marker expression in neural precursor cells

Reductionist model of adult neurogenesis to study neuronal development *in vitro*

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Protocol for culturing neurospheres from progenitor cells in the dentate gyrus of aged mouse hippocampus

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SUMMARY

The neurosphere assay is the gold standard for assessing the proliferative and differentiation capacities of neural progenitor cells (NPCs). Here, we present a protocol for isolating, propagating, and maintaining hippocampal neurospheres from adult and aged mice and differentiating cultured NPCs into neurons and astrocytes. We describe steps for establishing a heterochronic co-culture of neurosphere-derived cells with primary neurons. Using neurospheres from old animals enables investigation of the effects of aging on the development and differentiation of newborn neurons.

BEFORE YOU BEGIN

The neurosphere assay (NSA) is a widely used *in vitro* technique to isolate and determine the proliferative and differentiation potential of stem cells and NPCs in rodent models.[1–5] This approach facilitates the study of these complex processes by recapitulating *in vivo* events, while also reducing known and unknown confounding variables present *in vivo*.[6] Importantly, this technique allows for expansion of the number of dividing cells and establishment of a homogeneous reservoir that can be cryopreserved for future experiments. These features make the NSA particularly useful for investigating cellular and molecular drivers of age-related reduction of adult neurogenesis. Since the population of NPCs in the adult brain is low and further decreases with age, this poses a technical challenge for detection and quantification of neurogenesis events *in vivo* such as cell growth, differentiation, integration and death.

This protocol describes isolation and culturing NPCs from adult and aged animals to study differentiation and integration of adult-born NPCs. To harvest and establish neurospheres from mice ≥12 months old, it is necessary to account for lower numbers of actively dividing NPCs and less efficient enzymatic degradation of tissue of adult and aged brain. To overcome these issues, we developed and integrated an additional step allowing the initial dissociated cells to proliferate on a coated plate, so the rare progenitor cells reach confluence before replating as free floating neurosphere cultures. We also found that the proprietary enzymatic cocktail provided in the Neural Dissociation kit for Postnatal neurons from Miltenyi Biotec provides the best dissociation and cell viability outcome, allowing culturing of neurospheres from mice up to 18-months old. The combination of longer culturing times after plating and more efficient papain-based tissue dissociation allowed

us to develop a reliable protocol to grow, expand, and differentiate neurospheres from aged mice. This protocol has also been successfully used to generate NPCs from wild-type C57Bl/6 as well as transgenic Gli1CreERT2xAi9 mice. While the current protocol was designed and validated for generating neurospheres from aged mice, we also tested the protocol on early postnatal (day 10) and young adult animals (1–6 months) and were able to successfully establish neurospheres from these time points as well. Therefore, we consider that the current protocol can be utilized for isolating NPCs from a wide range of ages.

Institutional permissions

All animals were maintained according to the IACUC-approved protocols at the University of California, Davis. All animal experimentation must be approved by the institutional animal use and care committee and be conducted in accordance with institutional animal use and care guidelines. When working with live cells, it is essential to conduct all procedures under sterile conditions in a certified biosafety cabinet. Work involving primary cell lines and neural tissue should be approved by an institutional biosafety committee and performed in accordance with the Biosafety Level 2 (BSL2) guidelines.

Preparation of growth factors

1. Reconstitute bFGF and EGF with 0.1% BSA solution.
   a. Dilute bFGF and EGF at a concentration of 100 μg/mL.
   b. Make 20 μL aliquots of reconstituted growth factors.
   c. Freeze aliquots at −80°C for long term storage.

Note: Avoid freeze thaw cycles, keep defrosted aliquot at 4°C up to a week.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Monoclonal mouse IgG1 anti-GFAP clone N206A/8 (dilution 1:1,000)	NeuroMab	Cat#75-240; RRID: AB_2877343
Polyclonal rabbit IgG anti-β-Tubulin III/Tuj1 (dilution 1:500)	GenScript	Cat#A01627; RRID:AB_2622164
Monoclonal mouse IgG1 anti-Nestin clone 10c2 (dilution 1:500)	Santa Cruz	Cat#sc-23927; RRID: AB_627994
Recombinant monoclonal rabbit IgG anti-SOX2 clone EPR3131 (dilution 1:10,000)	Abcam	Cat#ab92494; RRID: AB_10585428
Polyclonal guinea pig IgG anti-DCX (1:1,000 dilution)	Millipore	Cat#AB2253; RRID:AB_1586992
Chemicals, peptides, and recombinant proteins		
(Z)-4-hydroxy Tamoxifen	Cayman Chemical	14854
Accutase	STEMCELL Technologies	07920
Animal-free recombinant human EGF	PeproTech	AF-100-15
Antibiotic-antimycotic (100X)	Gibco	15240096
B-27 supplement (50X), minus vitamin A	Gibco	12587010
B-27 supplement (50X), serum free	Gibco	17504044
Boric acid	Sigma-Aldrich	B0394
Bovine serum albumin	Sigma-Aldrich	A9418
Collagen, type 1 rat tail	Corning	354236
DAPI	Invitrogen	D1306
D-glucose (dextrose)	Sigma-Aldrich	G7021
DMEM/F12	Gibco	11320033
DPBS	Gibco	14080055
Ethyl alcohol, pure	Sigma-Aldrich	E7023
Freezing medium Cryo-SFM	Sigma-Aldrich	C- 29912
GlutaMAX supplement	Gibco	35050061
HBSS, calcium, magnesium, no phenol red	Gibco	14025092
HEPES	Gibco	15630080

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Horse serum	Gemini Bioproducts	100-508
Laminin mouse protein	Gibco	23017015
MEM	Invitrogen	51200-038
Microscope slides	Thermo Fisher Scientific	PO103
N-2 supplement (100X)	Gibco	17502048
Neurobasal medium	Gibco	21103049
Papain	Worthington Biochemical	LS003126
Paraformaldehyde solution, 8%	Electron Microscopy Sciences	1578100
PBS	Gibco	10010023
Poly-D-lysine	Fisher	P7405
Poly-L-ornithine	Sigma-Aldrich	P3655
ProLong Gold antifade mountant	Invitrogen	P36930
Recombinant human FGF-basic (154 aa)	PeproTech	100-18B
Sodium bicarbonate ($NaHCO_3$)	Sigma-Aldrich	S5761
Sodium pyruvate	Invitrogen	11360-070
Sodium tetraborate decahydrate	Sigma-Aldrich	S9640
Triton X-100	Sigma-Aldrich	T8787
Trypan blue solution, 0.4%	Gibco	15250061
Trypsin-EDTA	Invitrogen	25300-054
Tween 20	Sigma-Aldrich	P1379
Water sterile cell culture grade	Gibco	A1287301
Critical commercial assays		
Neural tissue dissociation kit (enzyme P)	Miltenyi Biotec	130-092-628
Experimental models: Organisms/strains		
Mouse: Ai9, both male and female, 1–18 months old	The Jackson Laboratory	RRID: IMSR_JAX 007909
Mouse: C57BL/6J, both male and female, 1–18 months old	The Jackson Laboratory	RRID: IMSR_JAX 000664
Mouse: Gli1-CreERT2, both male and female, 1–18 months old	The Jackson Laboratory	RRID: IMSR_JAX 007913
Software and algorithms		
GraphPad Prism	GraphPad	https://www.graphpad.com/scientific-software/prism/
Fiji (ImageJ)	NIH	https://imagej.net/
Microsoft Office	Microsoft	https://www.office.com/
Other		
15 mL Falcon centrifuge tube, sterile	Corning	352096
2 mL cryogenic vial	Corning	431386
24-well clear TC-treated multiple well plate	Corning	3524
3 mL transfer pipet, individually packed, sterile	Corning	357575
48-well clear TC-treated multiple well plate	Corning	3548
5 mL round-bottom polystyrene tube with cell strainer snap cap	Corning	352235
50 mL Falcon centrifuge tube, sterile	Corning	352070
CO_2 incubator, Heracell 150i	Thermo Fisher Scientific	50116047
Double edge razor blades	Electron Microscopy Sciences	72000
Freezing container	Thermo Fisher Scientific	5100-0001
German glass coverslips 12 mm diameter round	Neuvitro	GG-12–1.5
Glass Pasteur pipettes	Thermo Fisher Scientific	22-183632
Kimwipes delicate task wipers	Kimberly-Clark	06-666
McIlwain tissue chopper	Stoelting	51350
Petri dishes with clear lid, 100 mm	Fisherbrand	FB0875713
Petri dishes with clear lid, 60 mm	Fisherbrand	FB0875713A
Plastic discs for tissue chopper stage	Stoelting	51354
Phase counting chamber	Hausser Scientific	3200
Single edge razor blades	Electron Microscopy Sciences	71960
Tissue culture treated flasks, 25 cm^2	Fisher Scientific	10-126-1C
Whatman qualitative filter paper circles, 90 mm	Millipore	WHA1001090

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Light operating scissors straight, sharp/sharp	Fine Science Tools	14060-11
Dissector scissors, angled to side, sharp/sharp	Fine Science Tools	14082-09
Micro spoon	VWR	82027-526
Micro dissector spatula	Fine Science Tools	10087-12
Fine forceps, Dumont #5	Fine Science Tools	11251-20
Low power white light stereo microscope	Motic	K500

MATERIALS AND EQUIPMENT

Cell culture media and stock solutions

Dissection solution		
Reagent	Final concentration	Amount
HBSS	1x	250 mL
$NaHCO_3$	26 mM	0.55 g
d-Glucose (Dextrose)	30 mM	1.35 g
HEPES	2 mM	500 μL
Total		250 mL

Note: Filter-sterilize and keep at 4°C up to 6 months. For long-term storage keep at −20°C.

Dissection media		
Reagent	Final concentration	Amount
DMEM/F12	1x	494.5 mL
Antibiotic-Antimycotic	1x	5 mL
N2 supplement	1x	0.5 mL
Total		500 mL

Note: Keep the media at 4°C up to 2 weeks.

Media for NPCs maintenance		
Reagent	Final concentration	Amount
Neurobasal media	1x	48 mL
Antibiotic-Antimycotic	1x	0.5 mL
GlutaMAX	1x	0.5 mL
B27 supplement, minus vitamin A	1x	1 mL
bFGF	20 ng/mL	10 μL
EGF	20 ng/mL	10 μL
Total		50 mL

Note: Filter-sterilize and keep at 4°C up to a month.

⚠ CRITICAL: To increase stability of growth factors, only preheat the necessary volume of media for each day.

Media for NPCs differentiation		
Reagent	Final concentration	Amount
Neurobasal media	1x	48 mL
Antibiotic-Antimycotic	1x	0.5 mL
GlutaMAX	1x	0.5 mL
B27 supplement	1x	1 mL
Total		50 mL

Note: Keep the medium at 4°C up to 2 weeks. Add B27 supplement on the day of the differentiation experiment.

Media for Astrocyte plating and maintenance		
Reagent	Final concentration	Amount
MEM	1x	434 mL
Donor horse serum	10%	50 mL
30% w/v D-glucose solution in MEM	0.36%	6 mL
GlutaMAX	1x	5 mL
Antibiotic-Antimycotic	1x	5 mL
Total		500 mL

Note: Prepare 30% w/v % D-Glucose solution in MEM first. Filter-sterilize and keep at 4°C up to 4 weeks.

Neural plating media for primary hippocampal culture		
Reagent	Final concentration	Amount
MEM	1x	432.5 mL
Donor horse serum	10%	50 mL
30% w/v D-glucose solution in MEM	0.45%	7.5 mL
Sodium pyruvate	1x	5 mL
Antibiotic-Antimycotic	1x	5 mL
Total		500 mL

Note: Filter-sterilize and keep at 4°C up to 2 weeks.

Borate buffer		
Reagent	Final concentration	Amount
Sodium tetra-borate	45 mM	17.2 g
Boric acid	85 mM	3.1 g
Sterile water	1x	Up to 1000 mL
Total		1000 mL

Note: Filter-sterilize solution. Store buffer at 4°C for up to 6 months.

Poly-D-Lysine stock solution		
Reagent	Final concentration	Amount
Poly-D-Lysine	10x	100 mg
Borate buffer	1x	10 mL
Total		10 mL

Note: Filter-sterilize the stock solution before aliquoting. Aliquot in 1 mL aliquots and store in −20°C freezer.

⚠ CRITICAL: At the time of coverslip coating prepare 1 mg/mL poly-D-lysine in borate buffer by diluting 1 mL 10X aliquot in 9 mL borate buffer.

STEP-BY-STEP METHOD DETAILS

Dissociation and plating of dentate gyrus cells of adult or aged mouse

Timing: Reagent prep: 2 days; dissection: 2 h

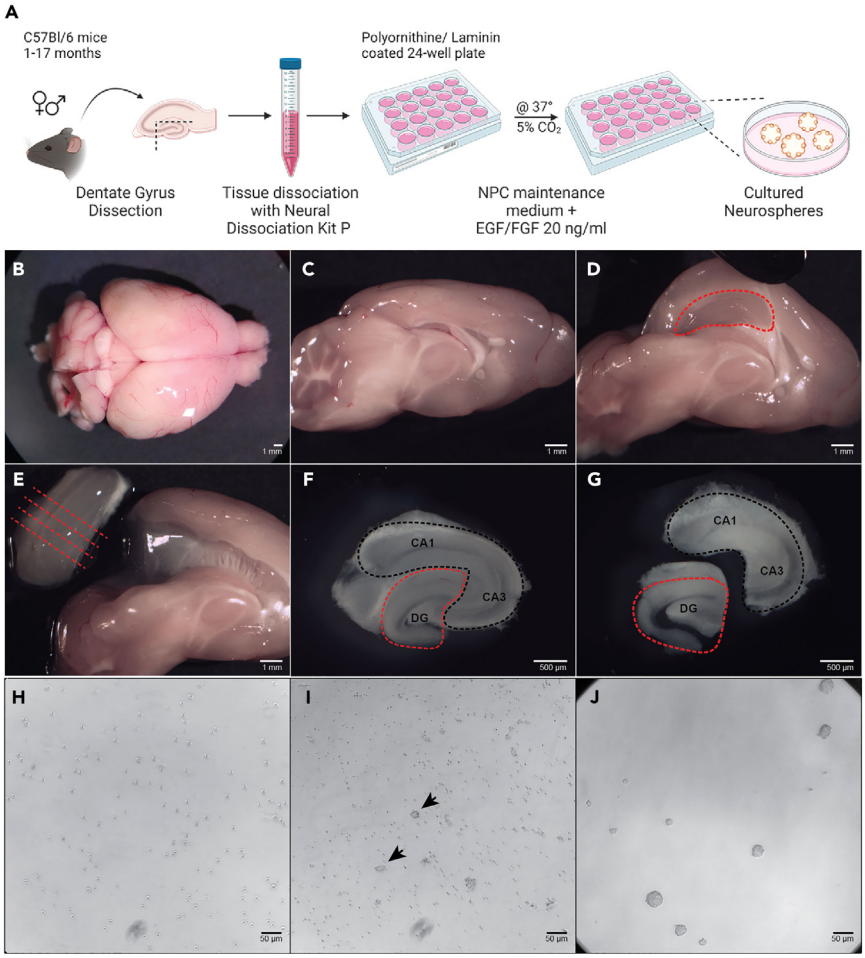

Figure 1. Neurospheres isolated from the dentate gyrus of the adult or aged mouse brain

(A) Workflow for isolation and culturing of neurospheres from adult or aged mouse dentate gyrus. Diagram created in BioRender. https://BioRender.com/p35h730.

(B) Brain is removed from the skull and placed in a dissection dish.

(C) Hemispheres are separated using a razor blade at the sagittal plane.

(D) Spatula is used to move the cortex from the midline to free the hippocampus.

(E) Isolated hippocampus before sectioning with the tissue chopper. Red dashed lines indicate the direction of sectioning.

(F) A slice of hippocampus after a tissue chopper. Red dashed lines show outline of dentate gyrus (DG), black lines, cornus ammonis (CA) regions CA1 and CA3.

(G) Microdissected DG separated from CA regions.

(H) Bright field image of dissociated DG cells plated after dissection.

(I) Formation of clusters of newly formed neurospheres (black arrows).

(J) Neurospheres formed after the first passage. Scale bar: 50 μm.

This step describes the procedure for brain dissection followed by digestion and trituration of tissue into single cells (Figure 1A).

1. Preparation before experiment.
   a. A day before the dissection, clean the dissection tools by washing them with mild soap and water.
      i. Place tools on a kimwipe to air dry.
      ii. Place tools and filter paper circles in the sterilization pouch and autoclave.
      iii. Put a single-edge, a double-edge blade, plastic discs for McIlwain tissue chopper stage and forceps in a beaker containing 70% ethanol, keep tools immersed for at least 5 min.

iv. Spray tissue chopper with 70% ethanol, paying special attention to the stage and the arm. Wipe ethanol with lint free kimwipe.
v. Use forceps to attach a double-edge razor blade and discs onto the tissue chopper.
vi. Set the slice section width to 125 μm. Spray the chopper again after the installation is finished, air dry.
vii. Transfer a single-edge razor into a new beaker with sterile water and keep it there before use.

b. Prepare coverslips.
i. Place glass coverslips in 2 M nitric acid solution and leave on the orbital shaker for 8–12 h.
ii. Using tweezers, remove the coverslips from the acid bath and wash at least 8 times with double-distilled water in a new clean container.

Note: Make sure the acid is washed off. You can check that by measuring pH of wash water.

iii. Use tweezers to place coverslips on kimwipes to dry.

Note: Coverslips should be completely dried before the next step otherwise they will stick to each other.

iv. Once dried, transfer coverslips to an autoclaving container.
v. Sterilize coverslips by autoclaving at a dry cycle.

Note: Coverslips can be prepared ahead of time, as long as the plates containing the coverslips are maintained in a sterile environment prior to use.

c. Coating solutions preparation.
i. Prepare poly-L-ornithine stock solution by dissolving it in sterile water to 10 mg/mL concentration.
ii. To make a working solution, dilute the stock solution with sterile water to make a final concentration of 20 μg/mL.

Note: Aliquot and store at −80°C up to 6 months.

iii. Prepare laminin working solution by diluting it in Neurobasal media to make a final concentration of 5 μg/mL.

Note: Thaw and make 50 μL aliquots of laminin, store at −80°C freezer. For better results, avoid repeated freeze/thaw cycles.

⚠ CRITICAL: Laminin should be slowly thawed in the 4°C fridge and should not be stored after dilution to a working solution.

d. Coverslip coating with Poly-L-Ornithine and Laminin.
i. Place 12 mm coverslips into a 24-well plate.

Note: Center the coverslips in the wells; they shouldn't touch the sides of the well, otherwise the coating solution might spread out outside the coverslip.

ii. Pipette 100 μL of 20 μg/mL poly-L-ornithine solution onto each coverslip.
iii. Incubate the coverslips in the solution for 8–12 h in the 37°C tissue culture incubator.
iv. Aspirate poly-L-ornithine solution and air-dry coverslips by keeping plate lid off in the biosafety cabinet.

Note: The 24-well plates with poly-L-ornithine-coated coverslips can be sealed with parafilm and kept at 4°C for a month for future use.

v. A day before use for culture, pipette 100 μL of 5 μg/mL laminin solution per coverslip.
vi. Keep the coverslips with laminin solution in the 37°C tissue culture incubator for 8–12 h before cell plating.

Note: Avoid drying out of laminin before cell plating as that might affect cell attachment and survival in downstream experiments.

e. Prepare and chill Dissection solution.

Note: Make 30 mL of Dissection solution for each mouse brain to be dissected.

i. Pour 20 mL of dissection solution into a 100 mm cell culture plate, and 5 mL into a 60 mm petri dish for each mouse.
ii. Place sterile filter paper circles on the bottom of the 100 mm petri dishes.
iii. Prepare a 15 mL tube per mouse by adding 3 mL of dissection solution.
iv. Place the dishes and tubes on the ice to cool the solution for at least 10 min.

Note: Filter paper on the bottom of the plate helps to keep the brain steady during the dissection.

f. Prepare Dissection and NPCs maintenance media on the day of dissection.
   i. Prepare at least 5 mL of Dissection media and 1 mL NPCs maintenance media with growth factors per mice for anticipated number of animals.
   ii. Put Dissection media into a water bath at 37°C to warm up before starting the dissection.
g. Prepare Enzyme mixture 1 from the Neural tissue dissociation kit before the start of the brain dissection.
   i. Mix 1900 μL of Buffer Y with 50 μL of Enzyme P from Miltenyi dissociation kit per mouse in 15 mL tube in the biological safety hood.
   ii. Place the solution into the water bath at 37°C to warm up.
h. Prepare Enzyme mixture 2 from the Neural tissue dissociation kit before the start of the brain dissection.
   i. Mix 20 μL of Buffer Y and 10 μL of Enzyme A from the commercial dissociation kit per animal in 1.5 mL tube.
   ii. Keep the tube on ice before use.

Note: We have found that 4–5 mice are the maximum that can be effectively harvested at the same time without affecting downstream results. If animals of different genotypes are used in the experiment it is advisable to use a different clean set of tools, fresh plates with dissection media and a new blade for the tissue chopper to be used for each group to limit possibility of cross contamination.

Note: Step 2–4 are performed at the bench that is thoroughly cleaned and sprayed with 70% ethanol before the start of the dissection.

2. Gross mouse brain dissection.
   a. Euthanize an adult or aged mouse by intraperitoneal injection of pentobarbital sodium solution, to the dose of >100 mg/kg according to an IACUC-approved protocol.
   b. Assess euthanasia by tail, toe and eye blink reflex 3–5 min after injection. In the absence of a reflex, decapitate the animal with large scissors placed just above the cervical region of the spinal cord.

c. Make a caudal-rostral cut into the skin with the same scissors from the base of the skull toward the nose to open the scalp.
d. Make a straight cut into the skull bone in between eye sockets and two short horizontal cuts on both sides of the skull base.
e. Using small-angled scissors, cut the skull in a caudal-rostral direction toward the nose to open the skull.
f. Slide a spoon spatula into the cut, moving it underneath the skull bone in between the bone and the brain and push it to the side to open the skull like a "book". Repeat with the other side.
g. Place the spatula underneath the brain moving from the front to back and flipping the brain out of the skull.
h. Put the whole brain in a 50 mL tube containing ice-cold dissection solution to wash out the blood.

3. Hippocampus isolation.
a. Transfer the entire brain to a 100 mm dish containing ice-cold Dissection solution (Figure 1B).
b. Make a sagittal cut with a single-edge razor blade through the central fissure to separate left and right hemispheres.
c. Place a hemisphere lateral side down (Figure 1C).
d. Place the spoon spatula in the fold between the cortex and the midbrain. Carefully push the cortex to the side exposing subcortical structures.
e. Flatten the cortex with a spatula while using another spoon spatula to hold it in place, hippocampus should be visible in the caudal part on the ventral surface of the cortex (elongated, C-shaped structure) (Figure 1D).
f. Use the micro dissector spatula to cut the hippocampus from underneath the cortex. Isolate hippocampi from both hemispheres (Figure 1E).

4. Slicing of the hippocampus and DG microdissection.
a. Use a sterile plastic transfer pipette to pick up each hippocampus with a drop of the dissection solution and gently place it on the tissue chopper stage, one at a time.

Note: Place it such that the hippocampal long axis is perpendicular to the chopper arm. Put the hippocampus at the center of the stage and a couple of millimeters away from the cutting edge of the arm. It is important to perform this step and slice the hippocampus quickly to prevent tissue from drying out as that would negatively affect cell survival.

b. Pull out the table release knob and switch the tissue chopper on.

Note: Tissue can also be cut by rotating manual operating knob.

c. After the entire hippocampus has been sectioned, turn off the chopper by pressing the off switch.

⚠ CRITICAL: Ensure that the chopper is turned off before retrieving the tissue slices, as retrieving them while the chopper is running poses a high risk of injury.

d. Use a sterile plastic transfer pipette with ~0.5 mL of dissection solution to pick up the tissue slices and transfer them into a 60 mm petri dish.

Note: Label the 60 mm plates with animal ID and/or genotype.

e. Place the dish with tissue slices under a dissection microscope. Identify the DG and CA regions (Figure 1F).
f. Use a spoon spatula to hold a slice and a micro dissector spatula to isolate the DG by cutting between CA1/CA2 and the upper blade of DG.
g. Make another cut between CA3 and the end of the hilus (Figure 1G).

h. Perform this procedure on all the slices.
i. Collect DGs in a 15 mL tube containing 3 mL ice-cold Dissection solution.

5. Tissue dissociation and NPC plating.

Note: At this step all DG cells are plated into the well. It is possible to have differences in the number of viable cells between the animals, it is mostly dependent on the dissection proficiency. However, it is possible to establish neurosphere culture even with very few viable NPC as these cells continue to proliferate and expand before replating. Another important contributor to the differences in the number of viable cells is the animal's age at the time of dissection. Cultures from older mice have less viable, proliferating NPCs and more tissue debris.

a. Take the tubes with isolated DGs to the biological safety hood.
b. Wait for tissue slices to sink to the bottom of the tube, then carefully aspirate the Dissection solution.
c. Add 1950 μL of pre-warmed dissociation solution per tube and incubate in a 37°C water bath for 15 min.
d. Invert the tubes 10 times every 5 min.

Note: While waiting for incubation, turn on the centrifuge and set it to 500 × *g*. Prepare round-bottom polystyrene tube with cell strainer tube, one per mouse, by pipetting 2 mL of prewarmed (37°C) Dissection media through the strainer into the tubes. Place NPCs maintenance media into a 37°C water bath to prewarm.

e. Pipette 30 μL of cold Enzyme solution 2 to each tube with cell suspension. This helps to minimize clumping of concentrated cell suspension after enzymatic tissue digestion.
f. Use a fire-polished glass pipette (~0.8 mm diameter) to gently triturate 10 times.

⚠ CRITICAL: Avoid making air bubbles as they can produce physical strain and rupture cell membranes.

g. Return tubes to the water bath to incubate for an additional 10 min.
h. Gently invert the sample 10 times every 5 min.
i. After incubation, use a P1000 micropipette to pass the cell suspension through a strainer into tubes with Dissection media.
j. Hold the pipette tip perpendicular to the mesh and slowly expel the solution, ensuring the entire volume is transferred.
k. Centrifuge the tubes at 25°C at 500 × *g* for 5 min.
l. Carefully aspirate the media without disturbing the cell pellet and gently resuspend cells in 3 mL of fresh prewarmed Dissection media.
m. Centrifuge the cell suspension again for 5 min at 500 × *g*.
n. Aspirate the Dissection media and resuspend cells in 500 μL of prewarmed NPCs maintenance media.
o. Remove laminin solution from the coverslips in the prepared plate.
p. Plate the cell suspension into the wells of the plate: 2 hippocampi from 1 brain into 1 well in a 24-well plate and put the plate into the incubator.
q. After 2 h of plating, remove all the media and replace with fresh prewarmed NPCs maintenance medium. Check for cell attachment under the light microscope.
r. Change one third to one half of the NPCs maintenance media every other day.

Note: The volume of the media change depends on the number of live and proliferating cells in the culture. Since the number of viable and actively dividing cells varies depending on the age of the mice from which the culture was isolated, the rate at which these cultures

metabolize nutrients is also different. The volume of media change should be based on the color of the media in the well.

6. Establishing primary neurospheres.
   a. Examine the cell culture under a light microscope every three days to monitor proliferation. When the NPC culture reaches 30–50% confluency, it is ready for replating.

   Note: Dividing cells should form clusters (Figure 1I) and subsequently migrate out.

   b. Prewarm NPCs maintenance media and accutase enzyme mix in the water bath to 37°C.
   c. Aspirate the media from the well and pipette 500 μL of accutase.
   d. Return the plate to the incubator for 5 min.
   e. Use P1000 to gently dislodge attached cells by blowing the solution along the bottom of the well. Collect the accutase solution with dissociated cells from the well into a 15 mL tube.
   f. Centrifuge the tube at 500 × *g* for 5 min at 25°C.
   g. Aspirate accutase solution without disturbing the cell pellet.
   h. Resuspend cells in 500 μL fresh prewarmed NPCs maintenance media and pipette them in the uncoated well of a 24-well plate.

   Note: At this stage we do not routinely perform cell count.

   i. Place the plate back into the tissue culture incubator.
   j. After the replating the cells should form spheroid-shaped primary neurospheres 1–3 days after replating (Figure 1J).

NPC cell culture maintenance procedure

Timing: Reagent prep: 1 h; passaging: 30 min

This step describes the procedure for neurosphere maintenance and passaging.

Note: It is recommended to perform passaging once every 7 days to keep neurospheres size around 50–100 μm. We recommend determining the optimal timing for passaging based on the size of the neurospheres. For the experiments presented in this protocol, passaging was performed approximately every 7 days. However, the exact timing may vary between cell lines, particularly if the cells are derived from transgenic animals with phenotypes that affect the cell cycle duration or NPC proliferation. While all neurospheres are composed of a mixed population of precursor cells at various differentiation stages, smaller size helps to keep more homogeneous cell composition. Diversity of stem, proliferating neural progenitor cells and postmitotic neurons and glia within spheroids increases with size since more differentiated cell types arise after longer time in culture. Also, cells in the center within bigger spheroids (>250 μm) suffer from poor gas exchange and receive less nutrients from the cell media. That might lead to cell death.[7] Cell passaging can be repeated weekly (Figures 2A–2C), resulting in an exponential increase in total cell number. Cells of desired passage numbers can be cryopreserved for future use as discussed in the next section. Neurosphere cell cultures can be isolated and maintained across a wide range of animal ages (Figure 3).

7. Dissociating neurospheres with an average diameter 50–100 μm into single cell solution.
   a. Prewarm NPCs maintenance media and accutase enzyme mix in the water bath to 37°C.
   b. Use a P1000 pipette to transfer contents of the well to be passaged into a 15 mL tube.

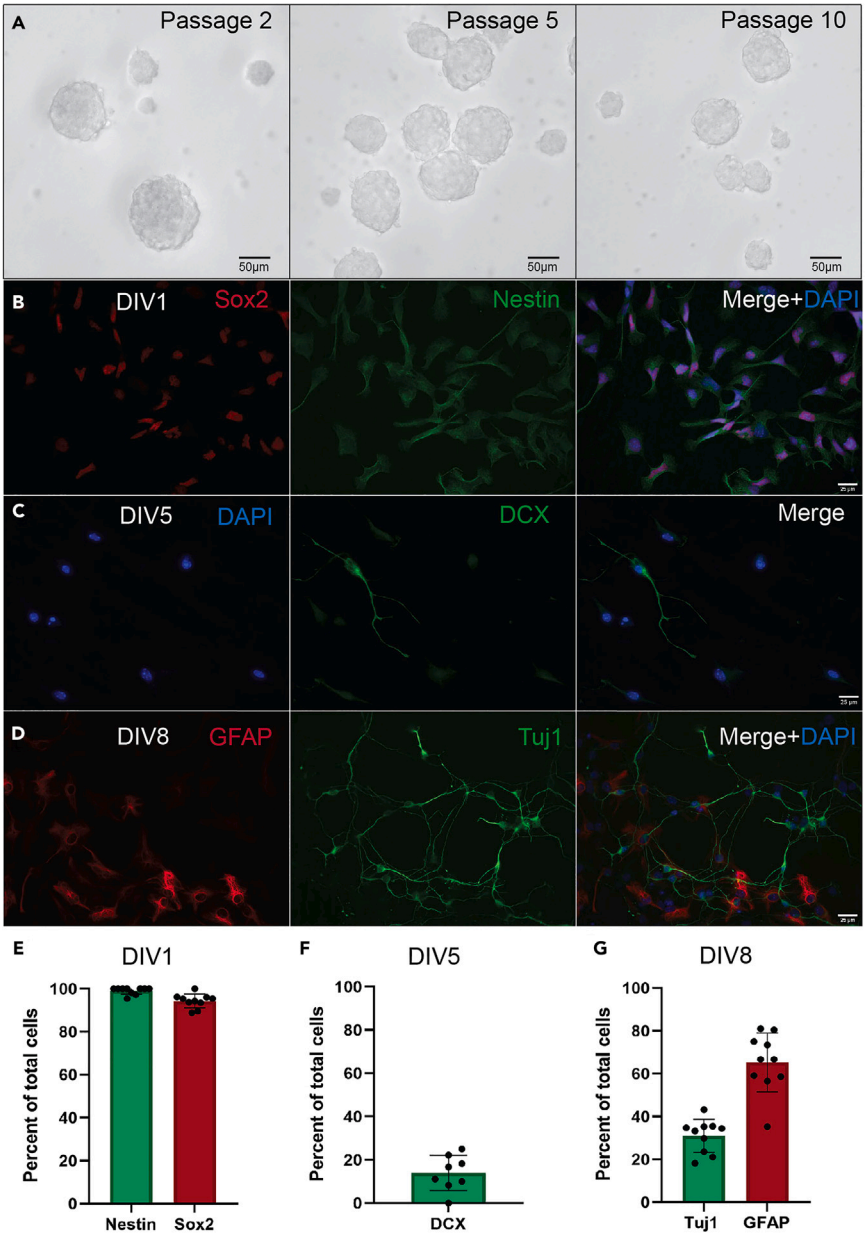

Figure 2. Adult-derived neurospheres exhibit neural progenitor properties

(A) Adult-derived neurospheres exhibit capacity for self-renewal. Neurospheres from the 11.5-month-old mouse continue to form after passaging cells 2, 5 or 10 times. All images are taken at DIV3 after plating. Scale bar: 50 μm.

(B) Dissociated NPCs from neurospheres derived from 11.5-months-old mouse at DIV1. The cells were immuno-stained with neural stem cell markers Nestin and Sox2. Scale bar: 25 μm.

(C) Neurosphere-derived NPCs from an 11.5-month-old mouse, passaged 4 times, were cultured for 5 days to induce differentiation (DIV5). The cells were immunostained with the immature granule cell marker doublecortin (DCX) and counterstained with DAPI. Scale bar: 25 μm.

(D) Dissociated NPCs from neurospheres derived from 11.5-months-old mouse at passage 4 after 8-day differentiation in culture (DIV8). The cells were immuno-stained with astrocyte marker GFAP and for neuronal marker Tuj1. Scale bar: 25 μm.

(E) Quantification of Nestin and Sox2 expressing cells at DIV1 post plating. Mean with ±SD.

(F) Quantification of immature DCX positive granule cells at DIV5 post plating. Mean with ±SD.

(G) Quantification of GFAP and Tuj1 expressing cells at DIV8 post plating. Mean with ±SD.

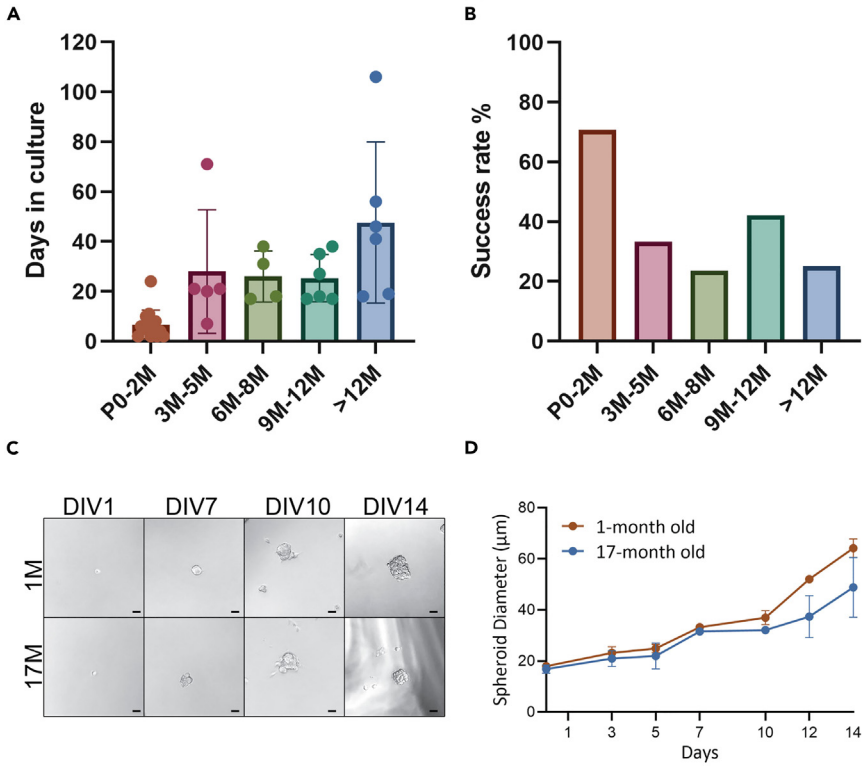

Figure 3. Stable neurospheres can be isolated from adult mice at different ages

(A) Time to the formation of neurospheres varies with age of animals, though stable cultures were established at several time points between 1 and 12+ months of age. Primary neurosphere is defined as a non-adherent cluster of cells ≥35 μm in diameter. Mean with ±SD.

(B) Success rate of establishing stable neurosphere cell lines decreases with age. Percent calculated by dividing the number of neurosphere cell lines established by the total number of animals dissected at each indicated time point.

(C) Bright-field images of growing neurospheres from young and aged animals, both passage 4 across 14 days *in vitro* (DIV14). Scale bar: 25 μm.

(D) Neurospheres from the young and aged animals grow in culture similarly in diameter up to DIV10. However, at DIV12 and 14 neurospheres from aged mice show lower spheroid diameter.

Note: If there are neurospheres attached to the bottom of the well, add fresh 500 μL of NPCs maintenance media to the well and use the P1000 pipette to gently dislodge these cells by pipetting up and down. Collect the media and combine with the rest of the well contents.

c. Centrifuge the tube at 500 × *g* for 5 min at 25°C.
d. Aspirate media without disturbing the cell pellet.
e. Add 500 μL of prewarmed accutase to the tube and use P1000 pipette to resuspend the pellet by triturating 3–4 times.
f. Incubate neurosphere suspension in the water bath for 5 min.
g. Centrifuge cells at 500 × *g* for 5 min at 25°C.
h. Aspirate the supernatant.
i. Resuspend the cells in 1 mL of NPC maintenance media with growth factors and calculate the cell number using a cell counter chamber.
j. Plate cells in 500 μL of NPCs maintenance media with growth factors at the concentration of $1x10^5$ - $5x10^5$ cells/mL in an uncoated 24-well plate.
k. Small neurospheres should be observed by the next day.
l. Change one third of the media every other day.

⚠ CRITICAL: It is important to monitor cultures frequently to determine the conditions of the neurospheres. The neurospheres should appear as round with light edges, translucent/not dark center and with small protruding cilia. Cell culture media should be pink colored. In case if the media appears yellow the frequency of media change should be adjusted (e.g., be everyday) to keep cells healthy.

Preparing a frozen stock of NPCs

Timing: NPC prep: 30 min; freezing: 2 days

During neurosphere passaging, cells can be frozen and stored in liquid nitrogen for future use, which is described here.

Note: Neurosphere culture can be frozen either dissociated into a single-cell solution or as spheroids. We tested both options (data not shown), and we found that freezing neurospheres as spheroids have better recovery rate, and we use this method for preparation of frozen stock in our experiments.

8. Prepare reagents and equipment for cryostorage.
   a. Prewarm Cryo-SFM media to 25°C.
   b. Prepare the freezing container rack by filling it with 100% isopropanol.
   c. Label cryovials with information about the neurosphere cell line, passage number and the date of freezing.
9. Collect neurospheres for long-term storage in liquid nitrogen tank.

Note: In our experiments we use one cryovial per well.

   a. Use a P1000 pipette to transfer free-floating neurospheres between 50-100 μm in diameter to be frozen into a 15 mL tube.
   b. Collect neurospheres at the bottom of the tube by spinning it at 500 × *g* for 5 min at 25°C.
10. Resuspend neurospheres in freezing media.
   a. Aspirate media without disturbing the cell pellet.
   b. Add 1000 μL of 25°C Cryo-SFM media to the tube and use a P1000 pipette to triturate and resuspend the pellet.

⚠ CRITICAL: Avoid introducing air bubbles.

   c. Transfer the cell suspension to labeled cryovial.
11. Freeze the neurospheres.
   a. Place the cryovial with neurospheres into a freezing container.
   b. Put the container with cells at −80°C freezer for at least 8 h.
   c. Transfer the freezing container to the liquid nitrogen tank for long-term storage.

Recovering neurospheres from a frozen stock

Timing: Reagent prep: 2 days; cell recovery: 30 min

This section describes the procedure for recovery of cryopreserved neurosphere cell lines.

Note: In our experience neurosphere cell lines can be successfully stored and recovered after 2 years of storage in the liquid nitrogen tank.

12. Prepare culture plates for cell recovery.
    a. Prepare a 24-well culture plate two days before the planned recovery by adding 300 μL of 20 μg/mL poly-L-ornithine in DPBS, using one well per sample.
    b. Place the plate into the incubator and leave the solution for 8–12 h.
    c. The next day aspirate poly-L-ornithine solution and air-dry in the coverslips by keeping plate lid off in the biosafety cabinet.
    d. Add 300 μL of 5 μg/mL laminin in PBS solution per well. Keep the laminin solution in a well until ready for cell plating, aspirate right before plating cells.
13. Retrieve cryovials with frozen neurospheres.
    a. Promptly place the cryovials into a water bath at 37°C.

    Note: Keep the cap of a cryovial above water when swirling the cryovial to prevent contamination from the water bath. Swirl the vial to thaw the contents evenly.

    b. When the cell solution is fully thawed, remove the cryovial from the water and spray thoroughly with 70% ethanol before placing it into the biological safety hood.
    c. Slowly in dropwise fashion add 1000 μL of pre-warmed NPCs maintenance media per cryovial. Carefully pipette up and down 1–2 times.
    d. Transfer cell solution to a 15 mL tube.
    e. Centrifuge it at 500 × *g* for 5 min at 25°C.
14. Replate recovered neurospheres into prepared plate.
    a. Aspirate supernatant.
    b. Resuspend cell pellet in 500 μL prewarmed NPCs maintenance media per tube.
    c. Remove laminin from the well.
    d. Add neurosphere suspension into a culture plate.
15. Plate and maintain recovered neurospheres.
    a. Check the plated cells under the light microscope 4–6 h after plating.

    Note: Some neurospheres should attach to the bottom of the plate and cells migrating out of attached spheroids should be visible.

    b. Change 200 μL of media for NPC maintenance every other day.

    Note: We routinely freeze neurosphere cultures and have observed some cell death during the thawing process and initial plating of frozen cells. However, based on our experience, cells that recover successfully from frozen stocks exhibit no noticeable differences in proliferation or differentiation compared to neurospheres that have not undergone freezing.

    Note: All experiments presented in this protocol were conducted using cells at passage 4. We avoided using cells from higher passage numbers, as previous studies have suggested that passaging neurospheres more than 10 times might lead to increased genetic instability.[8]

Plating neurosphere cells for differentiation

Timing: Reagent prep: 2 days; cell plating: 45 min

In this step, we describe differentiation of the neurosphere cells plated on the coverslips.

Note: When utilizing cells from the frozen stock, we follow the recovery procedure outlined in the previous section and allow the cells to grow for a minimum of three days before

dissociating and plating them for differentiation. In this experiment we used neurospheres from 11.5-month-old mice, passaged 4 times.

16. Prepare the reagents for the differentiation experiment.
    a. Two days before plating cells for differentiation, prepare Poly-L-Ornithine/Laminin-coated coverslips as described in dissociation and plating of dentate gyrus cells of adult or aged mouse section.
    b. Before starting the experiment, prewarm the NPC maintenance media to 37°C and accutase to 25°C.
    c. Check the neurosphere culture.

Note: Neurospheres should appear round with light bright edges with small protruding cilia and translucent/not dark center under the light microscope.

17. Dissociate neurospheres with an average diameter 50–100 μm into single cell solution as described in step 7 a-g.
18. Calculate plating density for cell plating.
    a. Add 1 mL of NPC maintenance media with growth factors.
    b. Use fire polished glass pipette to triturate neurospheres until they are dissociated into a single-cell solution, between 5 to 10 times.
    c. Calculate the cell number by mixing cell solution 1:1 with trypan blue and loading it into a cell counter chamber.
    d. Perform live/dead cell count under a light microscope.
    e. Calculate volume of the cell solution necessary to obtain desired plating density.

⚠ CRITICAL: Plating density should be optimized for different cell lines, typically, $5x10^4$-$1x10^5$ cells per 12 mm coverslip.

    f. Plate the desired number of cells onto precoated coverslips with 500 μL of NPC maintenance media and place in the incubator for 15 min for attachment.
    g. Check cell attachment at the microscope by gently shaking the plate, attached cells should not float.

Note: Visual inspection at this step can help assess cell attachment immediately after plating. A high number of floating, non-adherent cells at this stage may serve as an early indicator of unsuccessful plating. Possible reasons for this issue include coverslips not being freshly coated with polyornithine/laminin before the experiment, a high percentage of dead cells in the neurosphere culture due to inadequate maintenance, or excessive cell death caused by rough trituration during the dissociation step.

19. Maintain differentiating cell culture.
    a. Change media to the Differentiation media after 2 days *in vitro* (DIV) counting the day of plating as DIV1.
    b. Maintain differentiating cells by changing 200 μL of differentiation media in each well every other day.

Note: At early stages of differentiation (DIV1-3), the majority of cells express early neural stem/progenitor cell markers (Figures 2B and 2E). By DIV5, differentiated immature granule cells become detectable (Figures 2C and 2F), and by DIV7, a larger number of cells differentiate into mature granule cell neurons and astrocytes (Figures 2D and 2G).

Immunocytochemical staining for NPC, neuron, and astrocyte markers

Timing: Reagent and cell culture prep: 2–10 days; staining: 5 h

This section describes immunofluorescence-based assay to examine NPCs expression of molecular markers of the different differentiation stages of these cells.

20. Dissociate, plate and differentiate neurosphere cells as described in the previous section.
21. Fix differentiated NPCs for immunocytochemistry with paraformaldehyde solution.
    a. Prepare a fresh batch of 4% paraformaldehyde (PFA) by diluting 8% PFA from the ampoule with PBS.
    b. Add sucrose to the PFA to make a 4% solution by weight.
    c. Check pH, adjust to 7.4 if needed.
    d. Prewarm the solution in a 37°C water bath for 5 min before staining experiment.

    Note: Alternatively, a 4% PFA solution can be prepared in-house. Aliquoted PFA should be stored at −20°C and is good to use for up to 6 months after preparation. Avoid freeze-thaw cycles.

    e. At the desired day of NPC differentiation, aspirate the media and rinse coverslips with PBS.
    f. Add pre-warmed 4% PFA/4% sucrose solution.
    g. Incubate the coverslips in the fixative solution for 12 min at 25°C.
    h. Remove the fixative and wash coverslips with PBS 3 times, 5 min each.
22. Permeabilize cells for labeling of NPCs for cell marker expression.
    a. Use tweezers to remove each coverslip from its well and place it cell side up on a piece of parafilm inside a humidified chamber consisting of a tray with wet paper towel or a kimwipe.
    b. Add 100 μL permeabilization solution (0.25% Triton X-100 in PBS) for 10 min immediately after placing coverslips into a humidified chamber.

⚠ CRITICAL: Avoid drying of the coverslips, that can affect staining quality. If many coverslips are being stained at once, gently pipette 100 μL PBS per coverslip to prevent drying, aspirate PBS before applying permeabilization solution.

23. Block non-specific antibody binding.
    a. Wash coverslips with PBS, once for 30 s and then twice for 5 min.
    b. Incubate the coverslips in the blocking solution (5% BSA in PBS + 0.2% Tween-20) for at least 35 min at 25°C.
24. Primary antibody labeling of NPCs for cell marker expression.
    a. Use a blocking solution to dilute primary antibodies.

    Note: Optimal concentration of each primary antibody should be determined empirically.

    Primary antibodies can also be diluted in the solution consisting of 10% serum from the same species used to raise the secondary antibody and 0.25% Triton X-100 in PBS.
    b. Apply 100 μL of diluted primary antibodies per 12 mm coverslip, such as anti-Nestin and anti-Sox2 for neural stem/progenitor cells, anti-DCX for immature granule cells or anti-Tuj1 for neurons and anti-GFAP for astrocytes.

Note: Differentiation of neurosphere-derived NPCs can be stopped at any time. A high percentage of NPCs are expressing Nestin and Sox2 markers at early stages of differentiation between DIV1-3. For generation of differentiated neurons and astrocytes 7 days are recommended. Cells expressing immature granule cell marker DCX can be detected after 5 days of differentiation.

c. Incubate for 2 h at 25°C or 8–16 h at a 4°C fridge.
d. After incubation, remove antibody solution and wash coverslips with PBS 3 times for 5 min.

25. Secondary antibody labeling.
    a. Dilute secondary antibodies conjugated to fluorophore of choice in the blocking solution.

    Note: Secondary antibodies should be raised in a species different from the host species of the primary antibody.

    b. Put 100 μL per 12 mm coverslip and incubate for 1 h at 25°C in the dark.

    ⚠ CRITICAL: Keep cells protected from light in the following steps.

    c. Remove the antibody solution and wash coverslips with PBS 3 times for 5 min.
    d. Counterstain with DAPI diluted in PBS to 400 ng/mL for 3 min.
    e. Aspirate DAPI solution and wash once with PBS.
    f. Mount coverslips with a drop of hard-set mounting medium (e.g., ProLong Gold Antifade Mountant) onto glass slides.
    g. Keep the slides in a dark, dry place for at least 2 h before imaging.

    Note: NPC stained for Nestin and Sox2 (Figure 2B) at DIV1 show approximately >90% positive labeling of all cells (Figure 2E). Differentiated neurons and astrocytes are detected at DIV8 (Figure 2D), with Tuj1 marker expressed in roughly 30% (Figure 2G) and GFAP is expressed in 60% of total cells.

    Differentiated cells can also be analyzed by other quantitative methods such as immunofluorescence-based assays, qPCR and flow cytometry.

In vitro tamoxifen-induced recombination for conditional gene expression in neurospheres derived from transgenic mice

Timing: 4 days

This approach can drive conditional transgene expression in NPC cell lines by treating neurospheres with 4-hydroxytamoxifen (4-OH TM) (Figure 4B).

Note: 4-OH TM is considered a hazardous substance. Protective coat and gloves should be worn all the time handling the 4-OH TM. Any waste material must be disposed of in accordance with the national and local regulations.

We show how this protocol can be applied to NPC derived from Gli1CreERT2xAi9. Gli1CreERT2xAi9 mouse line has both (1) a tamoxifen (TM)-inducible form of Cre-recombinase driven by the Gli1- promoter, a gene that is active in neural stem and precursor cells, and (2) a loxP site before a stop codon before the Rosa26-tdTomato gene (Figure 4A). Administration of TM allows for the expression of tdTomato (tdTom) fluorescent protein in cells with the active Gli1 promoter and permanently labels their progeny (Figure 4C). This allows the identification and tracking of differentiating cells derived from transgenic neurospheres.

26. Preparation of 4-OH TM.
    a. Dissolve 5 mg 4-OH TM powder in 250 μL molecular grade ethanol to make 5 μM stock solution.
    b. Gently swirl until completely dissolved.

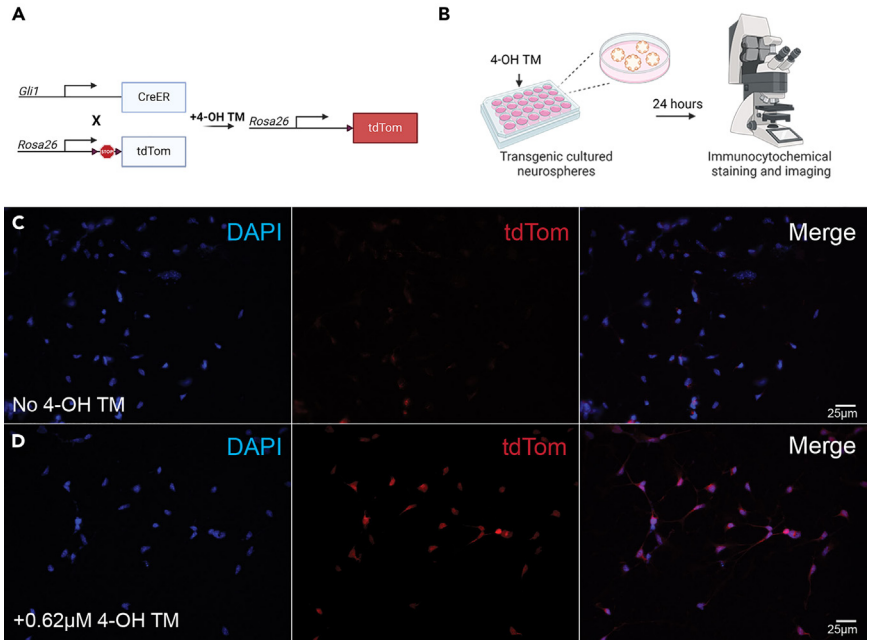

Figure 4. Neurospheres generated from adult or aged transgenic Gli1CreER x Ai9 mice

(A) A schematic diagram depicting how the Gli1CreER x Ai9 mouse line was generated. *In vitro* administration of 4-Hydroxytamoxifen (4-OH TM) to the neurospheres derived from these transgenic mice enables tracking of these isolated cells. Diagram created in BioRender. https://BioRender.com/g96h280.

(B) Workflow for *in vitro* induction of neurospheres derived from adult or aged Gli1CreER x Ai9 animals.

(C) Epifluorescent images of dissociated Gli1Cre x Ai9 neurospheres from an 11.5-month-old mouse, passage 4 without 4-OH TM treatment as a control. Cells were immunostained for tdTommato (tdTom) and DAPI.

(D) Epifluorescent images of dissociated Gli1Cre x Ai9 neurospheres treated with 0.62 μM 4-OH TM for 24 h. Cells were immunostained for tdTom and DAPI. Scale bars: 25 μm.

Note: 4-OH TM stock solution can be aliquoted and stored at −20°C protected from light for up to 6 months.

27. Prepare the 4-OH TM induction media by diluting the stock solution in freshly made NPC maintenance media just before starting the experiment.
    a. Adjust the final 4-OH TM concentration to 0.62 μM.
    b. Prewarm induction media in a water bath to 37°C.
28. Plating of transgenic NPCs.
    a. Plate $1*10^5$ cells per well cells per uncoated 24-well plate as described in step 7.
    b. Maintain neurospheres and allow them to grow for 3 days, or until they reach an average diameter of 50–100 μm.
    c. Transfer neurospheres from one well of 24-well plate into 15 mL tube.
    d. Centrifuge the tube for 5 min at 25°C at 500 × *g*.
29. *In vitro* 4-OH induction of NPCs.
    a. Aspirate the supernatant.
    b. Resuspend neurospheres in 500 μL of prewarmed NPC maintenance media with 4-OH TM.
    c. Pipette resuspended cells into an uncoated 24-well plate.
    d. Return cells into the tissue culture incubator at 37°C for at least 24 h.

Note: After the incubation period, NPCs are ready for downstream experiments, such as differentiation or co-culture with primary hippocampal neurons in the adult neurogenesis *in vitro* assay described in the next section.

The efficiency of 4-OH TM induction can be accessed by dissociating and plating NPCs onto glass coverslips as described in steps 17–18. NPCs treated with 4-OH TM show tdTom expression (Figure 4D) while NPCs that are treated with ethanol solvent with no 4-OH TM do not (Figure 4C).

We recommend performing 4-OH TM induction before each experiment on freshly expanded neurospheres. That provides a good quality cell labeling highlighting cell morphology. The tdTom fluorescent protein expression was observed in induced neurospheres and differentiated cells after passaging (data not shown). However, the labeling appears uneven throughout the cell.

Heterochronic co-culture of primary hippocampal neurons with neurosphere-derived cells

Timing: Media prep: 14 days; primary hippocampal culture: 7 days; co-culture: 9 days

This section describes the co-culture method that offers a simplified *in vitro* model of adult neurogenesis as NPCs differentiate and mature in the environment of the preformed neuronal network of primary culture.

Note: Here, the transgenic neurospheres from aged Gli1CreERT2xAi9 mice are dissociated and added to the early postnatal hippocampal culture at DIV7. In these steps, the preparation and maintenance of early postnatal mouse primary hippocampal culture as well as the introduction of neurosphere cells to co-culture are described.

Note: Astrocyte-conditioned media has been shown to promote neuronal health *in vitro*.[9] Prepare astrocyte conditioned media before postnatal mouse primary hippocampal culture. Conditioned media can be stored at 4°C for up to a month.

30. Prepare Poly-D-lysine coated flasks for primary astrocyte culture.
    a. Mix 4 mL of sterile cell culture grade water, 400 μL collagen and 80 μL poly-D-lysine (1 mg/mL) in a 50 mL tube for coating a 25 cm^2 plate.
    b. Pour 4000 μL of the coating solution per flask, tilt the flask side to side to evenly distribute the solution.
    c. Incubate the flask for 10 min in the biosafety hood.
    d. Aspirate the coating solution.
    e. Put the lid slightly askew and let the flask dry for 10–15 min.
31. Prepare reagents for mouse pup dissection.
    a. Mix up a digestion solution by combining 4900 μL HBSS and 100 μL papain into a 15 mL tube for each pup to be dissected.
    b. Place sterile filter paper circles on the bottom of the 100 mm petri dishes.
    c. Fill 100 mm petri dish 20 mL with cold Dissection solution.
    d. Place the dishes and tubes on the ice to cool the solution for at least 10 min.
32. Prepare and prewarm Astrocyte plating and maintenance media 15 mL media per flask.
33. Dissect mouse pup brain to harvest astrocytes.
    a. Anesthetize the postnatal day 0–5 mouse pups by placing them on a kimwipe-covered ice until not responsive.
    b. Place a new paper towel down and wipe each pup's head and neck with 70% ethanol well before rapidly decapitating using sterile scissors.
    c. Place a head into a petri dish filled with cold Dissection solution.
    d. Cut away skin and peel it back to expose the skull.
    e. Remove the brain by cutting into cerebellum and cutting along the middle of the skull: up and out with small-angled scissors.
    f. Use a spoon spatula to remove the brain and place it in the Dissection solution.
    g. Gently remove pia from the brain by pulling it off to the side of the brain using tweezers.

34. Perform cortical dissection.
    a. Use a microspatula dissector to remove cortex from both hemispheres.
    b. Cut cortex tissue into smaller pieces by pinning it down with tweezers and cutting with a micro dissector spatula.
    c. Using a sterile plastic pipette to transfer brain tissue to a clean 15 mL tube. Keep it on ice with 2–3 mL of dissection solution while finishing dissection of other pups.

Note: Use 1 tube per brain, that ensures more efficient cell dissociation.

35. Dissociate cortical tissue for astrocyte culture.
    a. Add 5 mL of digestion solution per tube.
    b. Incubate tubes for 15 min at 37°C in a water bath. Swirl tubes every 5 min.
    c. Once the incubation is complete, let the tissue settle on the bottom and carefully remove digestion solution. Avoid aspirating tissue.
    d. Add 5 mL of Astrocyte plating media to each tube and use a fire-polished Pasteur pipette triturate tissue 5–10 times.
36. Plate primary murine astrocyte culture for media conditioning.
    a. Centrifuge the tubes at 500 × *g* at 25°C for 5 min.
    b. Aspirate supernatant and resuspend the pellet in 1 mL of prewarmed Astrocyte media.
    c. Perform live/dead cell count by mixing 10 μL of cell solution and trypan blue in 1:1 ratio and quantify cell number using hemocytometer.
    d. Plate $2.25x10^6$ cells per 25 cm^2 poly-D-lysine-coated flask in 5 mL Astrocyte media, loosen cap slightly to allow gas exchange in the incubator.
    e. Perform complete media change 3 h after plating.
    f. Change total volume of media, 5 mL, the next day.
    g. Maintain astrocyte culture by changing a half of the media volume every three days until ~80% confluent.
37. Prepare purified astrocyte culture.
    a. Tighten the cap and place flasks with astrocyte culture onto a shaker set at 250 RPM at 37°C incubator for 8–12 h.
    b. Retrieve flasks from the shaker and check the culture under the microscope.

Note: Astrocytes should be attached to the bottom of the flasks while most of the undesired cells such as microglia and oligodendrocyte progenitor cells will be floating in the media. Foam in the culture media is normal.

38. Prepare flasks for replating purified astrocyte culture.
    a. Pre-warm 10 mL of Astrocyte media and warm up 1 mL Trypsin-EDTA per astrocyte flask to be re-plated.
    b. For each flask that will be replated, prepare 3 new cell culture flasks by adding 5 mL of Astrocyte media.
    c. Place new flasks in 37°C 5% CO_2 incubator to equilibrate for at least an hour.
39. Plate purified astrocyte into new plates.
    a. Remove media from the astrocytes and rinse the cells with 5 mL of sterile PBS by tilting the flask multiple times.
    b. Remove PBS and add 1 mL pre-warmed to 37°C Trypsin-EDTA.
    c. Incubate the flask for 5 min in the incubator. Check under the microscope for cell detachment.

Note: If cells are still attached, return to the incubator for another min.

    d. Add 9 mL prewarmed Astrocyte media to the flask. Use a 5 mL serological pipette to gently wash cells off the bottom of the flask. Transfer cell solution to a new 15 mL tube.

e. Centrifuge the cells at 500 × *g* at 25°C for 5 min.
f. Carefully remove supernatant.
g. Add 1 mL of Astrocyte media to the pellet and resuspend by triturating the pellet approximately 5 times or more if visible aggregates of cells remain in solution.
h. Perform live/dead cell count by mixing 10 μL of cell solution and trypan blue in 1:1 ratio and quantify cell number using hemocytometer.
i. Plate 3.5 × 10^5 cells per new flask with 5 mL of Astrocyte media.
j. Do a complete media change after 24 h after plating.
k. Replace half of the media every 3 days until cells are 80–90% confluent.

Note: Don't use astrocyte culture for media conditioning if the culture fails to reach 80–90% confluency after 3 days after replating.

40. Condition NPC differentiation media for co-culture with astrocytes.
    a. When astrocyte culture reaches 80–90% confluent, aspirate Astrocyte media and replace it with 12.5 mL of NPCs differentiation media.
    b. After 48 h collect astrocyte conditioned media and filter with a 0.22 μm filter.

Note: Label with date name and batch info: original culture date, split date. Store conditioned media at 4°C for up to one month.

41. Prepare for early postnatal hippocampal dissection.
    a. Prepare etched and sterile 18 mm coverslips as described in dissociation and plating of dentate gyrus cells of adult or aged mouse section.
    b. Place the coverslips into a 12-well plate and pipette 200 μL poly-D-lysine diluted in borate buffer to 1 mg/mL per 18 mm coverslip.
    c. Put the coverslips into the incubator for 8–12 h.
    d. Aspirate poly-D-lysine solution and rinse coverslips with sterile water 3 times.

Note: Keep the water on the plate after the last wash.

    e. Prepare and prewarm Neural plating media to 37°C.
    f. Mix up a digestion solution by combining 4900 μL HBSS and 100 μL papain into a 15 mL tube for each pup to be dissected.
    g. Place sterile filter paper circles on the bottom of the 100 mm petri dishes.
    h. Fill 100 mm petri dish 20 mL with cold Dissection solution.
    i. Place the dishes and tubes on the ice to cool the solution for at least 10 min.
42. Perform hippocampal dissection.
    a. Anesthetize the postnatal day 0–2 mouse pups by placing them on a kimwipe-covered ice until not responsive.
    b. Perform hippocampal dissection as described in step 3.
    c. Collect tissue to a 15 mL conical tube on ice with a transfer pipette. Keep it on ice with 2–3 mL of dissection solution while finishing dissection of all pups.
43. Dissociate primary hippocampal cells.
    a. Add 5 mL of digestion solution per tube.
    b. Incubate tubes for 15 min at 37°C in a water bath. Swirl tubes every 5 min.
    c. Let the tissue settle on the bottom and carefully remove the digestion solution. Avoid aspirating tissue.
    d. After incubation is complete, add 5 mL of prewarmed Neural plating media.
    e. Spin tubes at 500 × *g* at 25°C for 5 min.
    f. Aspirate the supernatant and
    g. Add 1 mL of Neural plating media.

h. Dissociate tissue with fire-polished glass pipette by triturating up and down 5–10 times, until no chunks of tissue are visible.
i. Perform live/dead cell count.

44. Plating primary hippocampal cells for co-culture.
    a. Retrieve poly-D-lysine coated coverslips from the incubator.
    b. Aspirate water.
    c. Plate cells at $3.5x10^4$ cells in 1.5 mL of neural plating media per 18 mm coverslip in 12-well plate.
    d. Place plates in an incubator for 4 h.
    e. After 4 h, completely remove Neural plating media and replace it with Astrocyte conditioned media.
    f. Perform media change every 2 days by removing 300 μL of media and adding 300 μL fresh Astrocyte conditioned media.
45. Introducing neurosphere cells to co-culture.
    a. On DIV7 of primary hippocampal culture collect and dissociate neurospheres as described in steps 16–18 of plating neurosphere cells for differentiation section.

Note: If using neurospheres from the transgenic inducible Cre mouse line, perform the 4-OH TM-induced recombination step, as outlined earlier, before adding the neurospheres to the co-culture. After dissociation, resuspend neurospheres in 1000 μL of Astrocyte conditioned media.

    b. Use fire polished glass pipette to triturate neurospheres until they are dissociated into a single-cell solution, between 5 to 10 times.
    c. Perform a live cell count by mixing cell solution with Trypan blue 1:1 and quantifying live cells in hemocytometer.
    d. Dilute cell solution to $5x10^4$ cells per mL of Astrocyte conditioned media.
    e. Slowly, in drop-wise fashion add 300 μL dissociated neurosphere cell solution into wells with primary neuronal culture.
    f. Return plates into the incubator.

Note: Under the light microscope neurosphere-derived cells could be distinguished by forming patches of more dense cells as they continue to proliferate for the first few days in co-culture. These NPS adhere to the bottom of the plate and don't form neurospheres in the co-culture system.

46. Co-culture maintenance.
    a. Perform media change every 2 days by removing 300 μL media and adding 300 μL fresh Astrocyte conditioned media.
    b. Maintain cell culture for another 7 days.

Note: The primary hippocampal culture in this method consists of neurons, astrocytes, and potentially other glial cell types such as microglia, oligodendrocytes, and oligodendrocyte progenitor cells (Figure 5). In our experiments we did not employ antimitotic agents like cytosine arabinoside (AraC) or 5-fluoro-2′-deoxyuridine (FUdR), which are often used to increase the neuron-to-glia ratio in primary neuronal cultures. Consequently, our cultures exhibited an astrocyte-to-neuron ratio of approximately 3:1, likely due to the unrestricted proliferation of glial cells.

Figure 6 shows the summary of experimental workflow, including time of initial DG culture plating to formation of primary neurospheres, NPC culture maintenance, preparation of the frozen stock of neurospheres, differentiation and co-culture establishment.

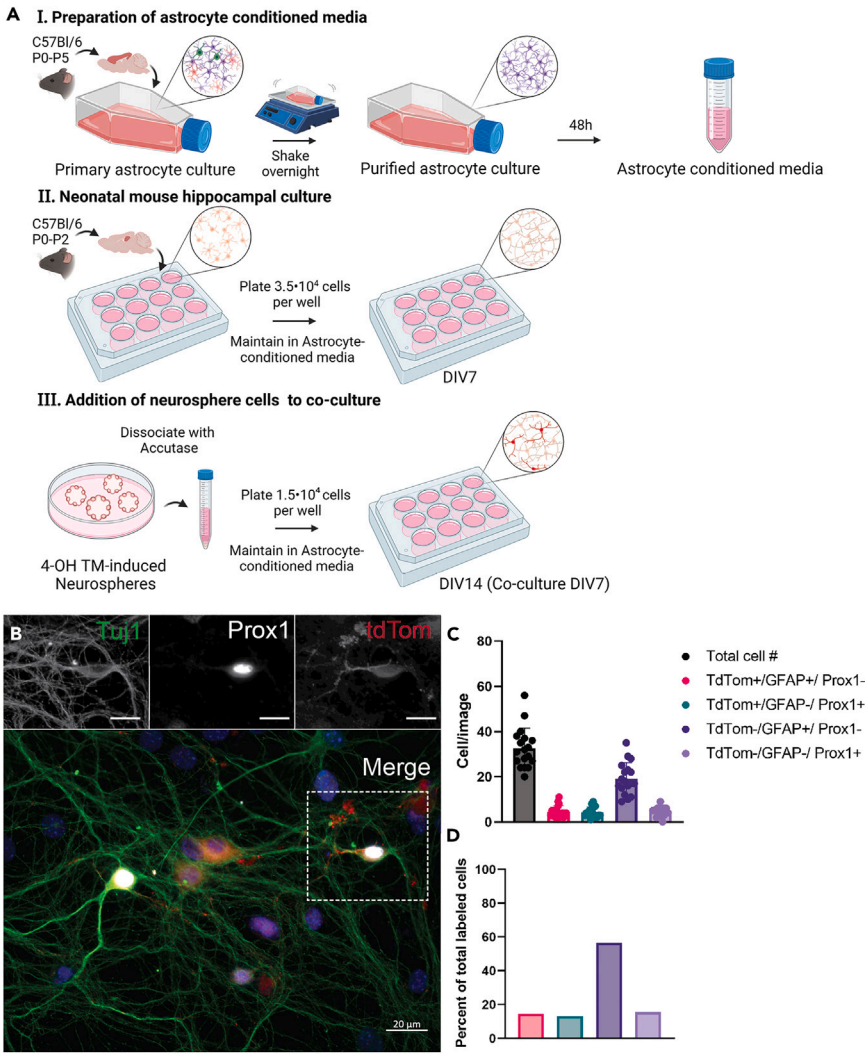

Figure 5. Heterochronic co-culture of granule cells differentiated from neurospheres derived from adult dentate gyrus with hippocampal cells from primary culture

(A) Flowchart of preparation of heterochronic co-culture of neurosphere-derived cells with primary hippocampal culture. Diagram created in BioRender. https://BioRender.com/o79f167.

(B) Epifluorescence image of DIV7 co-culture of granule cells and astrocytes differentiated from neurosphere cells derived from 11.5-month-old mouse, passage 4 and primary hippocampal cells. Culture is immunofluorescently labeled for Tuj1, tdTommato (tdTom) and Prox1. Nuclei are counterstained with DAPI. The upper images are from the rectangular area in the bottom image which is a merged image. Scale bar: 20 µm, inset scale bar: 5 µm.

(C) Co-culture composition shown by combination of tdTom labeling cells derived from neurospheres, astrocytic marker GFAP and marker for granule cells Prox1. Mean ± with SD.

(D) Percent breakdown of co-culture composition. The combinations of the expression of markers are used to identify specific cell type and origin: tdTom+/GFAP+/Prox1- : Astrocytes derived from neurosphere culture; tdTom+/GFAP-/Prox1+: Granule cells derived from neurosphere culture; tdTom-/GFAP+/Prox1-: Astrocytes from primary hippocampal culture; tdTom-/GFAP-/Prox1-: Unidentified cells from primary hippocampal culture.

EXPECTED OUTCOMES

The neurosphere assay protocol presented here allows isolation of NPCs from adult and aged mouse brains, expansion of the number of dividing cells and establishment of homogeneous reservoirs of cells that can be cryopreserved for future experiments. The ability of these cell lines for self-renewal and generation of differentiated progeny demonstrates that the NSA represents a sound approach for isolating and propagating NPCs from DG across all ages.

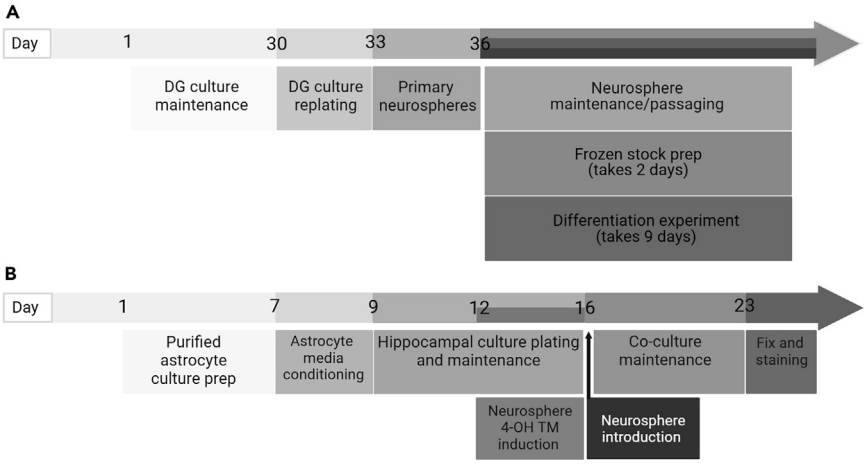

Figure 6. Summary of the experimental workflow timeline

(A) A visual depiction of the experimental process for establishing and maintaining neurosphere cultures from adult and aged mice. Each step is color-coded in boxes, aligning with a timeline illustrated as an arrow to indicate the duration required for each step. Preparation of the frozen stock and plating of cells for the differentiation can be performed alongside the passaging of the neurospheres for culture maintenance.

(B) A detailed workflow for the heterochronic co-culture system combining neurosphere-derived NPCs with early postnatal hippocampal cultures. The protocol for inducing transgenic neurospheres with 4-OH TM begins on day 3 (day 12 of the experiment) after plating the primary hippocampal culture. The induction process spans 4 days, during which neurospheres are maintained alongside the primary culture. On DIV7 (day 16 of the experiment) of the primary hippocampal culture, the induced neurospheres are introduced. The co-culture is then maintained for an additional 7 days before fixation and immunostaining. Diagram created in BioRender. https://BioRender.com/q59h461.

The current protocol was designed and validated for generating neurospheres from aged mice, we also tested the protocol on early postnatal and young adult animals and were able to establish neurospheres from these time points as well (Figure 3). Although culturing of the neurospheres from the aged animals over 12 months old is feasible it is important to note longer culturing times due to decrease in number of NPCs and their lowered proliferation capacity in aging (Figures 3A and 3B). Our data show that the mean time from DG culture plating to formation of primary neurosphere is 6.78 days for mice younger than two months while for animals between 9-12 months it takes on average 25.6 days (Figure 3A). Therefore, we consider that the current protocol can be utilized for isolating NPCs from a wide range of ages.

Multiple neurosphere cultures can be established from mice of the same age to serve as biological replicates and increase rigor of experimental applications. Moreover, neurosphere cultures generated from mice of both sexes provide a mechanism to investigate sex as a biological variable.

Isolated hippocampal progenitors cultured *in vitro* are multipotent and developmentally restricted cells that can give rise to new neurons and astrocytes. Cultured NPCs from the aged mice are expressing markers of neural stem/progenitor cells Nestin and Sox2 (Figure 2B) and differentiate into immature granule cells (Figure 2C), Tuj1-positive neurons, and GFAP expressing astrocytes (Figure 2D).

Co-culture of neurosphere derived NPCs with early postnatal hippocampal culture provides a platform to explore other components of the neurogenic niche (Figure 5A). The data shows that NPCs from transgenic neurospheres can survive and differentiate when co-cultured with other cell types (Figure 5B). Moreover, the NPCs have demonstrated to retain their multipotency by generating both granule cell neurons and astrocytes (Figures 5C and 5D). These results show that the co-culture method offers a simplified *in vitro* model of adult neurogenesis as transgenic NPCs differentiate and mature in the environment of preformed neuronal network of primary culture.

LIMITATIONS

A potential limitation of our protocol is low yield and long culturing times for neurospheres isolated from the aged animals. However, this could be attributed to decrease in the number of NPCs and increased quiescent state of these cells.[10–13] Long-term cell cultures like the one described in this protocol are prone to mycotic and bacterial infections, which can be remedied by good aseptic technique and the addition of antibiotics/antimycotic to the media.

Another important consideration is that the NSA might not be effective in detecting and isolating quiescent stem cells.[6] This is because quiescent stem cells are in the G0 phase of the cell cycle and therefore not actively dividing. By contrast, this NSA primarily selects for cells that are undergoing proliferation and allows for their expansion *in vitro*.

TROUBLESHOOTING

Problem 1

Bacterial or mycotic contamination of cell culture (related to steps 6–7).

Potential solution

Culturing of DG cells from aged mice requires longer culturing times. That increases risk of cell culture contamination. To prevent contamination of cell culture, include Antibiotic-Antimycotic to the culturing media. Also, it is advisable to freeze multiple vials of established neurospheres to ensure a supply of well-characterized cells. Freezing of at least 3 vials per cell line per for each passage number is recommended.

Problem 2

Low cell yield/viability after enzymatic tissue dissociation (related to step 5).

Potential solution

If enzymatic digestion is insufficient, increase incubation time while visually inspecting the progress of digestion. The same issue might be caused by use of excessive amounts of tissue. In this case dissociate maximum of 2 hippocampi in 50 μL of Enzyme P dissolved in prewarmed 1900 μL of Buffer X. Another reason for low cell yield and/or viability can be due to excessive trituration. Use fire polished Pasteur pipettes with not sharp edges. Make sure the opening is not too narrow ($\geq$0.5 mm in diameter). Pipette cells gently and avoid introducing air bubbles. Avoid harsh handling of cells during cell filtration. Do not scrape the mesh of the strainer when filtering cell suspension to avoid damaging the cells.

Problem 3

Failure to establish secondary neurospheres after passaging (related to step 7).

Potential solution

Timely passaging of neurospheres is a key to NPC maintenance. Failure of dissociated NPCs to proliferate after passaging can be the result of excessive incubation with accutase during the dissociation step. Another reason can be due to excessive trituration of neurospheres after accutase treatment. Use Pasteur pipettes with fire polished edges. Pipette cells gently and avoid introducing air bubbles.

Problem 4

Poor cell survival after *in vitro* 4-OH TM treatment (related to step 29).

Potential solution

TM can cause cell toxicity and prolonged incubation of neurospheres in media with 4-OH TM may lead to increase in cell death. Therefore, treat transgenic neurospheres with 4-OH TM for a maximum of 24 h.

Problem 5

Low number of neurosphere-derived cells in co-culture (related to steps 45–46).

Potential solution

NPCs from neurosphere culture retain proliferative capacity for the first few days in co-culture therefore they are plated at much lower numbers than primary hippocampal culture. In case too few neurosphere-derived cells are detected in co-culture increase the number of plated NPCs. Also, before introduction to NPC and co-culture, the hippocampal culture should be assessed. The cells in the primary culture should be healthy and exhibit developed axonal and dendritic branching at DIV7. Poor health and low cell number in the primary culture might negatively affect NPC survival and differentiation in co-culture.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Hwai-Jong Cheng (hjcheng@gate.sinica.edu.tw).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Olga Vafaeva (olga.vafaeva25@gmail.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate large datasets or code. Any additional information required to reanalyze the data reported in this paper will be made available on request from Hwai-Jong Cheng (hjcheng@gate.sinica.edu.tw).

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AUTHOR CONTRIBUTIONS

O.V. and P.N. performed the experiments, optimized assays, and analyzed the data. K.M., E.D., and H.-J.C. conceived the assays, supervised the experiments, and acquired funding. O.V. prepared the figures and wrote the paper under the supervision of K.M., E.D., and H.-J.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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