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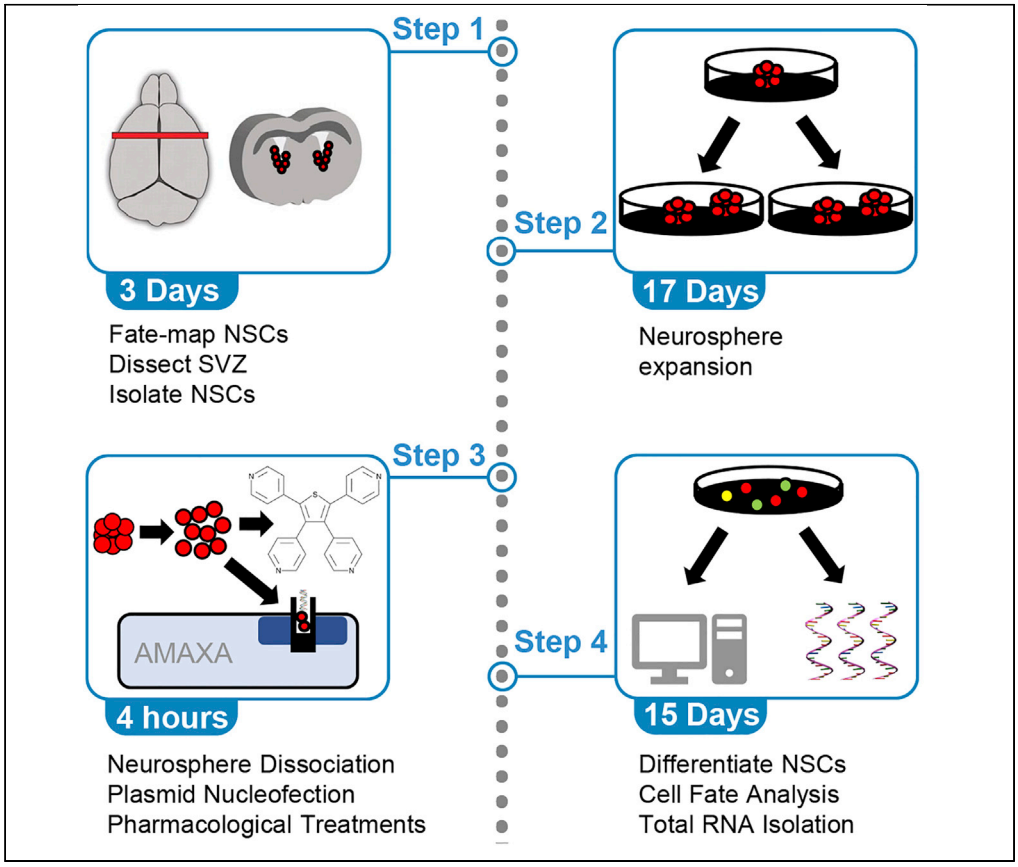
Protocol

Isolation and culture of neural stem cells from adult mouse subventricular zone for genetic and pharmacological treatments with proliferation analysis



Neural stem cells (NSCs) from the subventricular zone (SVZ) of the mouse brain can be expanded *in vitro* and grown as neurospheres, which can be stored long-term in liquid nitrogen. Here, we present a protocol for isolation and culture of NSCs from the adult mouse SVZ. We describe how to grow and expand primary NSCs to neurospheres, followed by differentiation and nucleofection/pharmacological treatments. Finally, we describe RNA extraction, EdU labeling of the cells, and immunofluorescent analysis to examine their proliferation.

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Highlights

Isolation of neural stem cells from the subventricular zone of adult mouse brains

Expansion of neural stem cells to neurospheres in suspension cultures

Dissociation of neurospheres for nucleofection and pharmacological treatments

Differentiation of neurospheres for RNA extraction and immunofluorescent analysis

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Isolation and culture of neural stem cells from adult mouse subventricular zone for genetic and pharmacological treatments with proliferation analysis

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SUMMARY

Neural stem cells (NSCs) from the subventricular zone (SVZ) of the mouse brain can be expanded *in vitro* and grown as neurospheres, which can be stored long-term in liquid nitrogen. Here, we present a protocol for isolation and culture of NSCs from the adult mouse SVZ. We describe how to grow and expand primary NSCs to neurospheres, followed by differentiation and nucleofection/pharmacological treatments. Finally, we describe RNA extraction, EdU labeling of the cells, and immunofluorescent analysis to examine their proliferation.

For complete details on the use and execution of this protocol, please refer to Radecki et al. (2020).

BEFORE YOU BEGIN

$Gli1^{CreERT2/CreERT2}$ mice were mated with the $Ai9^{Fx/Fx}$ reporter mice to generate $Gli1^{CreERT2/WT}$;$Ai9^{Fx/WT}$ ($Gli1^{CreERT2}$;Ai9) mice. This protocol describes the steps for harvesting and culturing Gli1 neural stem cells (NSCs) from 8 to 10 week old male and female $Gli1^{CreERT2}$;Ai9 mice after a single intraperitoneal injection of Tamoxifen which induces the expression of tdTomato from the Ai9 allele in Gli1 expressing cells. This protocol has also been successfully used to generate NSCs from wild-type C57bl/6 mice, as well as from mice with ages ranging from 1 month to 18 months. It should be noted that using older mice (~18 months age) decreases the yield of neurospheres and prolongs the time needed for their growth. In this protocol, we also describe the method for pharmacological treatment with GANT58, a small molecule Gli2 inhibitor, and genetic overexpression of Gli2 by nucleofection in NSCs. All animals were maintained according to the School of Veterinary Medicine IACUC protocols at the University of Wisconsin-Madison.

Tamoxifen and GANT58 preparation

Timing: 8 h total

1. Prepare 20 mg/mL Tamoxifen solution
   a. Dissolve 100 mg Tamoxifen in 5 mL sterile corn oil in a 50 mL conical polypropylene tube by shaking at 250 rpm in a 37°C incubator for 4–6 h, until the drug particles are dissolved and the solution appears clear. Keeping the solution at 37°C for extended periods may degrade the drug.

Note: Tamoxifen is stored at −20°C in the powder form until the expiration date on the vial. However, after dissolving in corn oil, the tamoxifen solution is stored at 4°C for a maximum period of 1 month.

2. Prepare 50 mM GANT58 solution
   a. Dissolve 196 mg GANT58 in 10 mL DMSO by vigorous mixing.
   b. Make 100 uL aliquots in microfuge tubes and store at −20°C for maximum 1 month.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Polyclonal Rabbit IgG anti NG2 (1:400 dilution)	Millipore	Cat# AB5320
Monoclonal Rat IgG2a anti RFP (5f8 clone) (1:1000 dilution)	Chromotek	Cat# 5f8
Monoclonal Mouse IgG2b anti MBP (Clone SMI 99) (1:100 dilution)	BioLegend	Cat# 808402
Goat anti-Mouse IgG2b Alexa 488 secondary Ab (1:1000 dilution)	Thermo Fisher Scientific	Cat# A21141
Goat anti-Rat Alexa 568 secondary Ab (1:1000 dilution)	Thermo Fisher Scientific	Cat# A11077
Goat anti-Rabbit Alexa 647 secondary Ab (1:1000 dilution)	Thermo Fisher Scientific	Cat# A21244
Chemicals, peptides, and recombinant proteins		
Tamoxifen	Cayman Chemicals	Cat# 13258
Corn oil	Sigma-Aldrich	Cat# C8267-500mL
GANT58	Cayman Chemicals	Cat# 14193
mEGF (Recombinant)	Stem Cell Technologies	Cat# 78016
bFGF (Recombinant)	Stem Cell Technologies	Cat# 78003
Heparin sodium salt	Sigma Aldrich	Cat# H3149
100× Antibiotic-Antimycotic	Gibco	Cat# 15240-062
0.25% Trypsin-EDTA	Gibco	Cat# 25200-072
Trypsin Inhibitor (from soybean)	Gibco	Cat# 17075-029
Hoechst 33342	Invitrogen	Cat# H3570
Goat Serum	Fisher Scientific	Cat# 10000C
Triton X-100	Fisher Scientific	Cat# 85111
Bovine Serum Albumin (BSA)	Fisher Scientific	Cat# BP9703-100
DMEM/F-12	Gibco	Cat# 11330-032
10× Phosphate Buffered Saline (PBS)	IBI Scientific	Cat# IB70165
Critical commercial assays		
Amaxa Mouse NSC Nucleofector Kit	Lonza	Cat# VPG-1004
NeuroCult Mouse & Rat Media	Stem Cell Technologies	Cat# 05702
NeuroCult Mouse & Rat Proliferation Supplement	Stem Cell Technologies	Cat# 05701
NeuroCult Mouse & Rat Differentiation Supplement	Stem Cell Technologies	Cat# 05703
Monarch Total RNA Miniprep	New England Biolabs	Cat# T2010S
Click-iT EdU Cell Proliferation Kit	Thermo Fisher Scientific	Cat# 10340
Experimental models: Organisms/strains		
Gli1^{CreERT2} Mouse: *Gli1*$^{tm3(cre/ERT2)Alj}$/J	The Jackson Laboratory	Stock# 007913
Ai9 Mouse: *ROSA26Sor*$^{tm9(CAG-tdTomato)Hze}$	The Jackson Laboratory	Stock# 007909
Recombinant DNA		
pCS2-MT_Gli2_FL	Addgene	Cat# 17648
Other		
Growth-Factor Reduced Matrigel (GFR Matrigel)	BD Biosciences (quality control testing by WiCell)	Cat# 354230
Amaxa Nucleofector 2b	Lonza	Cat# AAB-1001
100mm Deep Well Germination Dish	Midwest Scientific	Cat# 906
Mouse 1 mm coronal acrylic brain matrices	Braintree scientific	Cat# BS-A-5000C
50mL conical polypropylene tubes	Celltreat	Cat# 229421
12mm glass coverslips	Fisher Scientific	Cat# 64-0702
Cell culture treated 24-well plates	Dot Scientific	Cat# 229124
Fluoromount-G	Southern Biotech	Cat# 0100-01
Superfrost Plus Microscope Slides	Fisher Scientific	Cat# 12-550-15

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Tissue-Tek Accu-Edge Low Profile blades (tissue slicer blades)	Sakura	Cat# 4685
Premium dressing forceps	Fine Science Tools	Cat# FC-1002
Fine precision medium tipped forceps	Fisher Scientific	Cat# 12-000-157
SuperCut surgical scissors	Braintree Scientific	Cat# SCT-S 511
Mayo scissors	Fine Science Tools	Cat# 14010-15
Bonn scissors	Fine Science Tools	Cat# 14184-09
Vannas Spring Scissors – Angles	Fine Science Tools	Cat# 15008-08
Vannas Spring Scissors – Straight	Fine Science Tools	Cat# 15003-08
Scalpel handle #3	Fine Science Tools	Cat# 10003-12
#11 Scalpel blades	Fine Science Tools	Cat# 10011-00
Dumont Forceps	Fine Science Tools	Cat# FC-5043
Flat blade membrane forceps	Fisher Scientific	Cat# 09-753-50
Low power white light dissecting microscope	VWR	Cat# 490015-820

MATERIALS AND EQUIPMENT

Dissection media

Reagent	Final concentration	Amount
DMEM/F-12	1×	49.5 mL
100× Antimycotic-Antibiotic	1×	0.5 mL
Total	n/a	**50 mL**

Store at 4°C for up to 1 month

⚠ CRITICAL: Keep the dissection media on ice during the dissection.

Alternatives: DMEM or RPMI can be substituted for DMEM/F12. Penicillin-Streptomycin, containing 10,000 units/mL penicillin and 10,000 μg/mL of streptomycin, can be substituted for antimycotic-antibiotic. Antimycotic-antibiotic solution contains 25 μg/mL of Amphotericin B in addition to penicillin-streptomycin.

NSC Proliferation Media

Reagent	Final concentration	Amount
Neurocult Basal Media (Mouse & Rat)	1×	443 mL
Neurocult Proliferation Supplement	1×	50 mL
100× Antimycotic-Antibiotic	1×	5 mL
Mouse recombinant EGF – 10 μg/mL	20 ng/mL	1 mL
Mouse recombinant bFGF – 10 μg/mL	10 ng/mL	0.5 mL
Heparin – 0.2% w/v	0.0002%	0.5 mL
Total	n/a	**500 mL**

Store at 4°C for up to 1 month

⚠ CRITICAL: Thaw the Neurocult Basal Media, Proliferation Supplement, and Antimycotic-Antibiotic (Anti-Anti) to room temperature and combine them at room temperature. DO NOT warm the media in a 37°C dry/water bath. Room temperature (21°C–25°C) is recommended to preserve growth factors.

Note: Smaller aliquots of NSC Proliferation Media can be made, typically 50–100 mL, as this is sufficient for two to five 100 mm plates of NSC and will minimize growth factor degradation and loss.

NSC differentiation media		
Reagent	Final concentration	Amount
Neurocult Basal Media (Mouse & Rat)	1×	444.35 mL
Neurocult Differentiation Supplement	1×	50 mL
100× Antimycotic-Antibiotic	1×	5 mL
Mouse recombinant EGF – 10 μg/mL	2 ng/mL	100 μL
Mouse recombinant bFGF – 10 μg/mL	1 ng/mL	50 μL
Heparin – 0.2% w/v	0.0002%	0.5 mL
Total	n/a	**500 mL**
Store at 4°C for up to 1 month		

⚠ CRITICAL: DO NOT warm the media in a 37°C dry/water bath. Warming up to room temperature is recommended to preserve growth factors.

Note: Smaller aliquots of NSC Proliferation Media can be made, typically 50 mL, as this is sufficient for four 24-well tissue culture plates of NSC and will minimize growth factor degradation and loss.

GANT58 (Gli2 Inhibitor) 1000× Stock Solution		
Reagent	Final concentration	Amount
GANT58	50 mM	10 mg
DMSO	100%	509.6 μL
Total	n/a	**510 μL**

Note: GANT58 solution can be made in advance and stored at −80°C in smaller aliquots for up to 1 year.

Immunofluorescent labeling buffer solution		
Reagent	Final concentration	Amount
Bovine Serum Albumin (BSA)	1%	100 mg
Triton X-100	0.25%	25 μL
10× PBS	1×	1 mL
Distilled water	n/a	8.975 mL
Total	n/a	**10mL**
Make fresh and keep on ice or refrigerate at 4°C to be used within 24 h.		

STEP-BY-STEP METHOD DETAILS

Fate-mapping and harvesting NSCs from the adult mouse SVZ

Timing: 30 min for injection, 8 h reagent prep, 3 days total

In this step, *Gli1*^{CreERT2}*;Ai9* mice are injected with Tamoxifen to fate-map the Gli1 expressing NSCs, and three days later, the NSCs are harvested from the SVZ.

1. We pool the SVZ from 2 mice of similar age, matched sex, and genotype for a single dissection and NSC culture. Littermates are ideal.

Note: The following steps are for mice harboring a tamoxifen inducible CreERT2 allele and LoxP flanked genetic elements to initiate recombination *in vivo* prior to harvesting the cells for *in vitro* culture.

2. 5mg Tamoxifen intraperitoneal (i.p.) injection, 3 days prior to SVZ dissection
   a. Use a 1mL tuberculin syringe with a 27G needle
   b. Fill the syringe with tamoxifen and wipe the tip of the syringe before inserting the needle on the syringe.
   c. Perform i.p. injection with 250 µL (5 mg) of 20 mg/mL Tamoxifen per mouse, 3 days before the NSC harvest.

   Note: The typical dose of Tamoxifen is 100 mg/kg live weight. However, we examined the recombination efficiency with i.p. injections of 1 mg–6 mg tamoxifen and found that 5 mg Tamoxifen ensures recombination of the floxed allele in adult mice of C57bl/6 and swiss webster background strains with weights ranging from 20 g to 60 g, without any toxic effects on neural stem cells. We have also used this dosage in multiple CreER and floxed mouse lines (Radecki et al., 2020; Samanta et al., 2015, 2021).

3. Clean the gross dissection tools with 70% ethanol and allow to air dry. (Figure 1A).

4. For tools used in dissecting the brain and slicing (Figure 1B):
   a. Immerse 9 tissue slicer blades (feather cryostat blades), a mouse 1 mm coronal acrylic matrice and forceps in a beaker containing ~400 mL of 70% ethanol with lint free tissue paper (Kimwipes) lining the bottom to prevent damage to the tools. The beaker should be placed on ice to cool down the instruments.
   b. Rinse the tools in ice cold RPMI media in a 250 mL beaker with Kimwipes lining the bottom, before touching the brain tissue with them. RPMI is used as a more cost-effective alternative to DMEM/F12 (no difference was found using either solution). Anti-Anti is not necessary in the wash solution, as the ethanol and wash solutions are changed between harvest groups.

   Note: 6 animals is the maximum we have found that can be harvested into 3 plates at the same time without affecting downstream results. If NSCs are to be harvested from more than 6 mice in 1 day, it is recommended to change the ethanol and wash solution for all tools and the acrylic brain matrice after each harvest (2 mice). This ensures the tools remain clean and effective between harvests.

   c. Leave the tools in ice cold RPMI media when not in use.

5. Prepare and chill Dissection Media to 4°C (Figure 1C).
   a. 200 mL for washing tools and an additional 25 mL for the SVZ pooled from 2 mouse brains.
   b. Place 22 mL of dissection media into a 100 mm cell culture plate, and 3 mL into 35 mm cell culture plate for each pair of mice.
6. Prepare NSC proliferation media on the day of dissection
   a. Prepare enough NSC Proliferation media with growth factors for anticipated number of plates.

   Note: Each plate contains the SVZs from 2 mice in 20 mL of NSC proliferation media

7. Brain dissection and sectioning.
   a. Perform cervical dislocation or place the mouse in a CO_2 chamber for euthanasia.
   b. Spray 70% ethanol on the dorsal surface of the body beginning just caudal to the ears and moving rostrally to the nose.
   c. Separate the head from the body with a cut at the level of the atlanto-occipital joint using a large curved scissor (7 cm blade) placed immediately caudal to the ears.

   Note: Slight deviations rostrally or caudally will not affect the final generation of neurospheres.

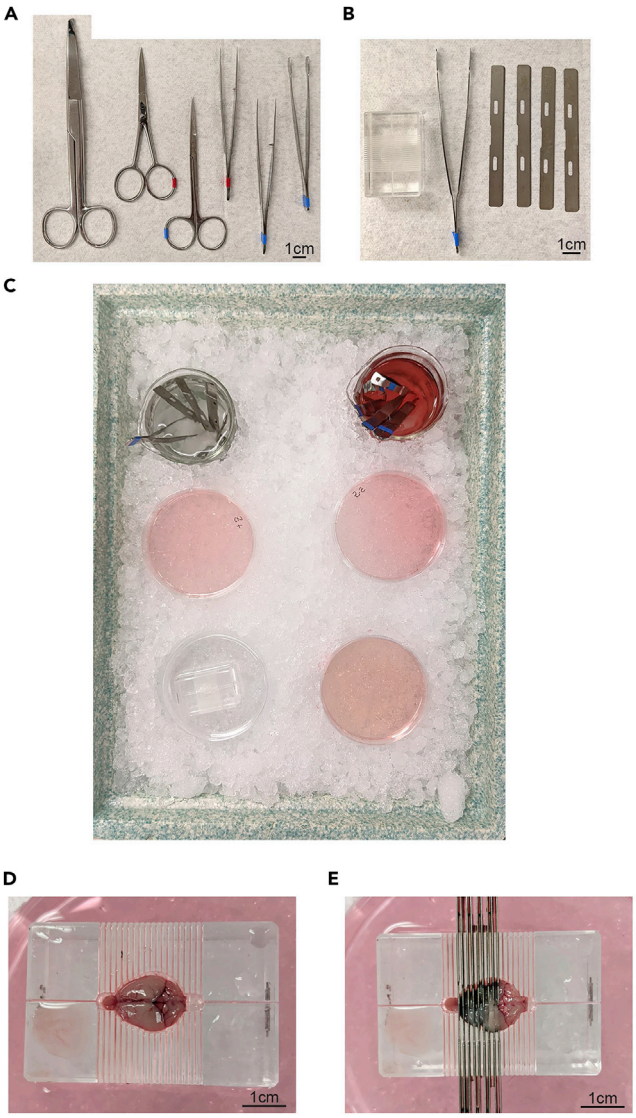

Figure 1. Preparation for dissection and slicing the brain

(A) Tools utilized for gross dissection and brain removal, from left to right: Large scissors for cervical dislocation, medium scissors for skin removal, small scissors for skull dissection, pointed tip forceps (×2) for skull removal, flat tip forceps for brain removal.

(B) Acrylic brain matrice and tools for slicing the brain and transferring the slices.

(C) Beaker and plate setup on ice for cooling tools and dissection media.

(D) Mouse brain placed in a coronal acrylic brain matrice with the dorsal surface facing up.

(E) Brain with tissue slicer blades placed every 1 mm from the olfactory bulbs to the anterior edge of the hippocampus.

d. Cut the skin from the dorsal surface of the head with a scissor.

e. Make a midline incision through the bone on the dorsal side of skull from the cut surface at the base of the skull to the orbits, using a small scissor (2 cm blade).

Note: Use pointed tip forceps to lift the skull flap on either side of the incision to expose the brain

f. Remove the brain from the skull using a flat tip forcep and place it in the coronal acrylic brain matrice with the dorsal surface of the brain facing up (Figure 1D).

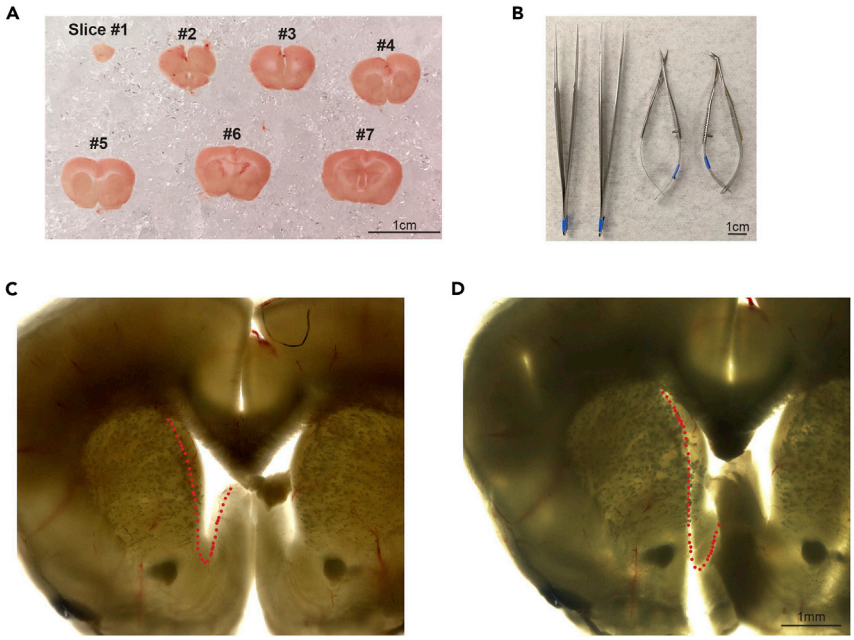

Figure 2. Slicing the brain and dissecting the SVZ

(A) 1mm thick brain slices numbered from rostral (#1) to the caudal slice (#7).

(B) Tools for SVZ microdissection including 2 pairs of fine point non-serrated forceps with straight and angled iris scissors.

(C) Enlarged image of brain slice #4 showing the speckled appearance of the striosomes in the striatum and the SVZ outlined by the red dotted line.

(D) Image showing the dissected SVZ with the incision from the ventral surface of the brain along the lateral and medial edges of the SVZ, outlined by the red dotted line.

g. Place blades, 1 mm apart, in the matrice, starting from the brain-olfactory bulb interface to the rostral end of the dorsal hippocampus (Figure 1E).

Note: Blades should be kept in ice-cold RPMI and the matrice should be kept on ice before use. It is critical that the blades and matrice are cold and moist, as warm or dry tools will cause the brain slices to affix to them resulting in tearing/damage to the slices. Slices are only obtained from the forebrain (approximately 8–9 mm, i.e. 8–9 slices), and the remaining brain is discarded.

h. Starting from the rostral end, lift each blade and transfer the brain slice to the chilled dissection media in a 100 mm dish kept on ice.

Note: Transfer each brain slice from the blade to the media using the pre-cooled flat tip forcep. Collect all brain slices up to the dorsal hippocampus (Figure 2A). DO NOT collect the subgranular zone (SGZ) of the dorsal hippocampus.

8. SVZ micro-dissection and dissociation.
   a. Place the 100 mm dish with 1 mm brain slices under a dissecting microscope
   b. Identify the brain slices where the lateral ventricles on both sides are separated from each other.

Note: In the caudal slices, the lateral ventricles connect in the midline. There are typically 3–4 slices that contain easily identifiable lateral ventricles in each hemisphere, not joined in the midline.

c. Dissect the SVZ from the brain slices with fine tip forceps and angled spring scissors with a 3–4 mm cutting edge (Figure 2B)
d. Incise a 1–2 mm wide layer of tissue bordering the lateral ventricles including the ventral apex, the medial wall, and the lateral wall of the ventricle in each slice (Figure 2C, pre-dissection, Figure 2D post-dissection)

Note: The striatum lies lateral to the lateral wall of the ventricle and can be identified by the speckled appearance of the striosomes under the microscope. The tissue between the striatum and the lateral ventricle is dissected for the SVZ (Figures 2C and 2D).

e. Place the dissected SVZ in the chilled dissection media in a 35 mm dish kept on ice.

9. Mince the dissected SVZ tissue into <1 mm thick pieces.
   a. A #11 scalpel blade and fine tip forceps work well for mincing the tissue.
   b. Instead of holding the tissue with the forceps, pin the tissue with the tip of the scalpel blade, and use the forceps to pull the tissue towards the sharp edge of the scalpel blade for mincing.

Note: It is not critical to generate the smallest piece possible, rather the goal is to cut the SVZ tissue to allow downstream digestion to occur more efficiently.

10. Collect the minced SVZ tissue in 50 mL conical tubes using a regular p1000 pipette tip and wash the bottom of each 35 mm plate with the last 1mL of dissection media to dislodge as many pieces of the SVZ as possible.
    a. Spin the tissue at 300 *g* for 5 min at 4°C.
    b. Aspirate the supernatant.
11. Resuspend the tissue in 1mL of pre-warmed 0.25% Trypsin-EDTA at 37°C.
    a. Incubate the tissue in a 37°C water bath for 5min.
12. Add an equal volume (1 mL) of 1 mg/mL Trypsin Inhibitor solution.
    a. Use a 3 mL syringe with 25G needle to dissociate the tissue into cells, taking care not to introduce air bubbles while triturating.
    b. Spin the cells at 500 *g* for 5min at 4°C.
13. Aspirate the supernatant and gently resuspend the cell pellet in 1 mL NSC Proliferation Media. Transfer the cells to a deep 100 mm plate with NSC Proliferation Media (total 20 mL) and incubate at 37°C with 5% CO_2.

Note: 20 mL media is essential to provide enough space for neurospheres to grow in suspension without touching the bottom of the plate. Deeper plates are recommended to avoid overfilling and prevent spills during handling. Alternatively, T25 or T175 cell culture flasks can be used with twice the recommended volume of media.

Primary neurosphere growth and supplementation

Timing: 7 days total

This step is for initial growth and expansion of primary, fate-mapped SVZ neural stem cells.

14. Allow the dissociated cells to expand for 48 h without disruption.
15. After 48 h, add fresh 40 µL of 10 µg/mL EGF (20 ng/mL final), 20 µL of 10 µg/mL bFGF (10 ng/mL final), and 20 µL of 0.2% Heparin (0.0002% final) directly to each dish and swirl gently.
16. After 3–4 days, clusters of cells or neurospheres are visible.

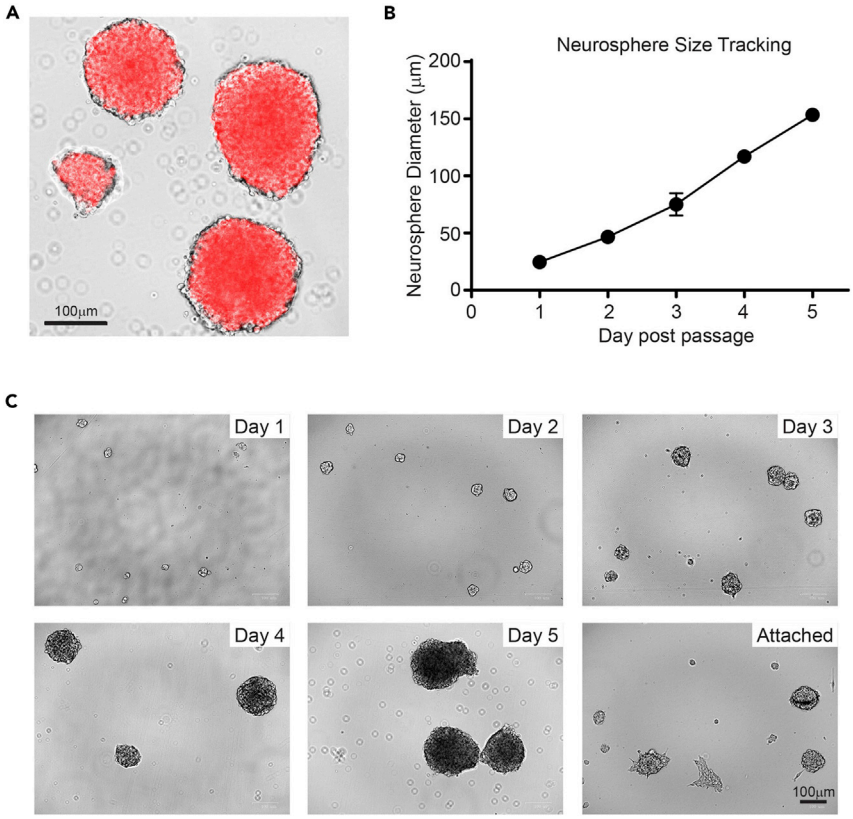

Figure 3. Growth of neurospheres

(A) RFP expressing fate-mapped neurospheres imaged 5 days after dissociation of tertiary neurospheres.

(B) Graph of the diameters of secondary neurospheres during the first 5 days after dissociation shows that neurosphere growth rates are consistent across experiments. The size of neurospheres were 24.3 ± 2 μm on day 1, 46.7 ± 0.9 μm on day 2, 75.3 ± 4.1 μm on day 3, 116.6 ± 0.7 μm on day 4, and 153.7 ± 0.9 μm on day 5. N = 3 independent cultures, data ± SEM.

(C) Images of secondary neurospheres comparing their size at each day after dissociation. On day 5, neurospheres begin to show a dark center which expands as they grow larger with a necrotic center. The bottom right is an example of neurospheres attached to the bottom of the plate, and beginning to differentiate.

Note: Allow primary neurospheres to grow for 7 days, or until they reach an average diameter of 150 μm. The media is not changed in the first 7 days to allow the neurospheres to grow without any disturbance.

Note: There will be some cell debris in the culture, but neurospheres are identifiable as bright spheres with well-defined edges. In case of neurospheres derived from fate-mapped NSCs, they should be uniformly positive for the fluorescence marker as shown in Figure 3A. Trouble-shooting 1

Neurosphere passaging

Timing: 1 h prep, 30 min active passaging

This step is for dissociating neurospheres with an average diameter ~140–160 μm into single cells.

17. Warm the NSC Proliferation media to room temperature.
    a. Take an aliquot of NSC Proliferation media and add growth factor supplements

18. Warm 0.25% Trypsin and 1 mg/mL Trypsin Inhibitor in a 37°C dry/water bath.
19. Remove the plates with neurospheres from the incubator and collect 10 mL of media with neurospheres in a 50 mL conical tube, with a sterile 10 mL serological pipet.
    a. Dislodge the attached neurospheres by gently pipetting the remaining 10 mL of media in the plate, repeatedly ~2–3 times.
    b. Add the remaining 10 mL of media containing the dislodged neurospheres to the previous 10 mL media in the 50 mL conical tube.
20. Spin the neurospheres at 300 *g* for 5 min at 4°C to form a pellet.
21. Aspirate the supernatant and add 1 mL Trypsin to the pellet followed by needle trituration.
    a. Incubate the dissociated neurospheres in a 37°C dry/water bath for up to 5 min.
    b. Add an equal volume (1 mL) of 1 mg/mL Trypsin inhibitor and swirl gently to mix.
    c. Dissociate the neurospheres into a single cell suspension by needle trituration in a 50 mL conical tube with a 3 mL syringe and 25G needle.

Note: Minimizing air bubbles at all steps will help ensure NSC survival. The presence of some bubbles will not cause a significant reduction in NSC survival. However, an excess of bubbles or froth (equal to 1/3 or more of the total volume of the cell mixture) will negatively impact downstream results.

22. Spin the cells at 500 *g* for 5 min at 4°C.
    a. Aspirate the supernatant and resuspend the pellet in 1 mL NSC Proliferation media.

23. Depending on the stage of passage, the neurospheres can be split using the following ratios (# starting plates:# final plates)
    a. Primary to secondary neurospheres = 1:1
    b. Secondary to tertiary neurospheres = 1:3
    c. Tertiary to quaternary neurospheres = 1:3

Note: Although neurospheres can be expanded for additional passages, we do not passage past the tertiary neurosphere stage. This minimizes the selection pressure on neural stem cells, preserves their *in vivo* characteristics and at the same time provides us enough cells for *in vitro* experiments.

    d. Dissociated neurospheres can also be counted and plated at specific densities. Figure 3B shows the growth rate for tertiary neurospheres after plating 1×10^6 cells from secondary neurosphere cultures. Troubleshooting 2

24. Plate cells in 20 mL of NSC Proliferation media in a deep 100 mm plate.

Figure 3C shows images of neurospheres growing during the proliferative phase after the first passage, as well as an example of attached neurospheres with differentiating cells. It is important to passage neurospheres when they reach 150 μm diameter as larger neurospheres can become necrotic if allowed to expand excessively. Troubleshooting 3

Optional: Neurospheres can be frozen and stored in liquid nitrogen, in the proliferative stage. It is recommended to freeze secondary neurospheres that are highly proliferative. Three days after splitting primary neurospheres, collect secondary neurospheres from one 100 mm plate by centrifuging them at 300 *g* for 5 min at 4°C. Resuspend the cells in 2 mL Freezing Media (NSC Proliferation media+10% DMSO), and freeze 1ml aliquots following standard cell freezing protocols. Upon thawing, discard the supernatant containing the freezing media after centrifugation at 300 *g* for 5 min at 4°C and then plate the cells in NSC proliferation media. The cells can be passaged after 72 h following the above protocol. This method has been successful for all mice including genetically modified mice.

Neural stem cell plating, differentiation, and treatment

Timing: 2 h prep over 3 days, 30 min differentiation, up to 14 days total for differentiation

In these steps, proliferating neurospheres are dissociated into single cells, plated onto coated dishes, and differentiated for up to 14 days with or without pharmacologic or genetic manipulations.

25. For differentiation, the NSCs are plated on 12 mm glass coverslips in 24-well plates. Coating the coverslips with growth factor reduced Matrigel (GFR-Matrigel) takes at least 21 h as described below:
    a. Etch the glass coverslips for 6 h by immersing them in 1 M hydrochloric acid at 37°C.
    b. Rinse the coverslips twice in 70% ethanol at room temperature.
    c. Incubate the coverslips in 70% ethanol for 6–12 h in a 120–200 rpm shaker at room temperature.
    d. To dry the coverslips, place the individual coverslips at a 45° angle, by resting the edge of the coverslip against the wall of the well in a 24-well plate, for at least 12 h or overnight.

    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.

    e. Coat the wells of the 24-well plates directly with Growth Factor Reduced Matrigel (GFR-Matrigel) for total RNA extraction or coat the etched coverslips for immunofluorescent analysis, a day before NSC dissociation.
    f. 1mg of GFR-Matrigel is sufficient to coat one 24-well plate.

    Note: This is equivalent to 42 μg of GFR-Matrigel per well or 0.52 mg/cm^2 in a 24-well plate. (Total surface area/well of a 24-well plate=1.9cm^2). 1mg of GFR-Matrigel is also sufficient for one 6-well, one 12-well, one 48-well, or one 96-well plate.

    g. Thaw the GFR-Matrigel on ice, then mix with 12 mL of cold media (RPMI, DMEM, or any buffered media).
    h. Add 500 μL of GFR-Matrigel/media mixture to each coverslip/well and then shake the plate to ensure that the media covers the entire coverslip/well.

    Note: Use a pre-chilled micropipette tip to dilute the matrigel and to add the diluted solution to each well.

    i. Place the plates in the biosafety cabinet for 3–12 h at room temperature to allow the GFR-Matrigel to polymerize.
    j. Following polymerization, the plates containing GFR-Matrigel can be stored for up to 2 weeks at 37°C. However, using these plates within 48 h of preparation is recommended.
    k. Immediately before plating the cells, aspirate the GFR-Matrigel from the well, and do not allow the GFR-Matrigel to dry completely. It is not necessary to rinse the wells after aspirating, however rinsing will not compromise plating or growth of cells.

26. Dissociate the proliferating neurospheres as in steps 17–22.
    a. Incubate the neurospheres in 0.25% Trypsin at 37°C for up to 5min.
    b. Add an equal volume of trypsin inhibitor to inactivate the trypsin.
    c. Dissociate the neurospheres into a single cell suspension by needle trituration in a 50 mL conical tube with a 3 mL syringe and 25G needle.

    Note: Gently pass the cells through the 25G needle, taking care not to introduce air bubbles while triturating. However, a few bubbles will not reduce survival of NSCs significantly. An

excess of bubbles or froth (equal to 1/3 or more of the total volume of the cell mixture) will negatively impact downstream results.

d. Spin the dissociated cells at 500 *g* for 5 min at 4°C.

27. Gently resuspend NSCs in 1 mL NSC Proliferation media by pipetting the pellet ~3 times.
28. Count cells and determine cell density.
    a. Typically, one 100 mm plate of tertiary neurospheres will generate 4–6 × 10^6 total cells.
    b. 1.5 × 10^6 cells are necessary for plating in a 24-well plate (0.63 × 10^5 cells/well) to ensure adequate cell numbers per well.
    c. Dilute cells as needed to obtain 12 mL cell suspension with 1.5 × 10^6 cells in NSC Proliferation media.

Optional: DNA plasmids can be introduced into NSCs while they are in a single cell suspension before plating on Matrigel coated surfaces. This step is performed after cell counting as the cell number is critical for successful nucleoporation. This procedure utilizes the Amaxa Nucleofector 2b machine (Lonza cat# AAB-1001), the Amaxa Mouse NSC Nucleofector kit, and plasmids of interest e.g. pCS2-MT_Gli2_FL for over-expression of full length Gli2 protein. Following the manufacturer's instructions in the Nucleofector kit, use 3 × 10^6 cells for a 24-well plate (1.25 × 10^5 cells/well) and 10 μg of plasmid DNA for each reaction, as mentioned below in steps 29–32. The higher number of cells is necessary to overcome cell death observed in ~40%–50% nucleofected cells.

29. Following the manufacturer's instructions, add 0.5 mL nucleofector supplement to 2.25 mL of the Cell Line Nucleofector Solution V, provided in the Amaxa Mouse NSC Nucleofector kit, to make the Mouse Neural Stem Cell Nucleofector Solution. This combined solution is stable for three months at 4°C.

Note: Ensure media are at room temperature including: (1) Mouse Neural Stem Cell (NSC) Nucleofector Solution; (2) NSC Proliferation Media

Note: Plasmids of interest can be just thawed and do not need to reach room temperature

30. Determine the cell number to obtain 3 × 10^6 total cells for a 24-well plate (1.25 × 10^5 cells/well), assuming all the wells will be nucleofected with the same plasmid.

Note: The following calculations are for one reaction used for nucleofection of all cells in one 24-well plate. This protocol cannot be scaled up for nucleofection of more than one 24-well plate. Although four nucleofection reactions can be performed in parallel, more reactions can delay the procedure, increasing cell death in subsequent steps.

31. Prepare the appropriate number of cells for nucleofection and spin to pellet, and during the cell pelleting, prepare the Mouse NSC Nucleofector Solution with the necessary plasmid mixtures.
    a. Place 3 × 10^6 cells in a 1.5 mL microfuge tube.
    b. Pellet the cells by centrifuging at 150×*g* for 5 min at 4°C.
    c. In a separate 1.5 mL microfuge tube, add 100 μL of the Mouse NSC Nucleofector Solution for each 24-well plate of cells, and/or plasmid. For the nucleofection of 3 × 10^6 cells, add 10 μg of plasmid to the Mouse NSC Nucleofector Solution and mix by pipetting.

Note: The reaction can be scaled down to 1.5 × 10^6 cells by adding 5 μg plasmid to 100 μL of Mouse NSC Nucleofector Solution, and for 0.75 × 10^6 cells by adding 2.5 μg plasmid to 100 μL Mouse NSC Nucleofector solution.

Note: Mouse NSC Nucleofector Solution volume is kept constant; however the amount of plasmid can be scaled linearly with the number of cells.

d. Turn on the machine and set the Amaxa Nucleofector Program to A-33 or A-033, depending on the version of the machine.

32. Resuspend the pelleted cells in the Plasmid + Mouse NSC Nucleofector solution and proceed to the nucleofection step as quickly and accurately as possible.
    a. Aspirate the supernatant from pelleted cells.
    b. Resuspend the cell pellet with 100 μL of the Plasmid + Mouse NSC Nucleofector solution mixture using a p200 pipette with a filter tip by gently pipetting 3–5 times or until the cells are resuspended in a homogenous opaque solution.
    c. Use the plastic pipette provided in the Nucleofector Kit, transfer the cell mixture from the microfuge tube to the bottom of a cuvette.

Note: Avoid bubbles and ensure that the cell mixture completely covers the bottom of the cuvette.

    d. Place the cuvette in the Amaxa Nucleofector machine and run program A-33 or A-033.
    e. Immediately add 500 μL of NSC Proliferation Solution at room temperature to the cell mixture. Then use the plastic pipette from step 32c to transfer the cells from the cuvette back to the microfuge tube that was used to pellet the cells in step 31c

Note: To increase cell survival, minimize the amount of time the cells are suspended in the Nucleofector Solution. Some amount of cell death is unavoidable; hence we begin with a higher number of cells. Working quickly with no more than 4 reactions at a time results in at least 60% cell survival. Troubleshooting 4.

33. For 3 × 10^6 cells, add 600 μL solution containing the nucleofected cells to 11.4 mL of NSC Proliferation Media, mix by gently pipetting and plate 500 uL of the cell suspension in each well of a 24-well plate.

Note: For 1.5 × 10^6 cells, resuspend the 600 uL of nucleofected cells in 5.4 mL of NSC Proliferation Media and plate 500 uL per well for total 12 wells. This resuspension can be scaled down depending on the number of cells used.

34. Allow cells to adhere overnight (8–16 h) before changing the media to NSC Differentiation media.
35. For pharmacological treatment of cells e.g., a small molecule Gli2 inhibitor, GANT58:
    a. Treat the cells with the drug, 3–4 h after plating or when >90% of cells have attached to the matrigel coated wells containing 0.5mL NSC differentiation media in each 24-well plate.
    b. Use 50 μL NSC Differentiation media per well to dilute GANT58 for more accurate pipetting
        i. For one 24-well plate: Dilute 12 μL of 50 mM GANT58 (500 μM or 1000×) or 12 μL of DMSO (vehicle) in 1.2 mL NSC Differentiation Media.
        ii. Add 50 μL GANT58 or vehicle to 450 μL of NSC Differentiation media in each well of a 24 well plate (final concentration 50 μM GANT58 or 0.1% DMSO), gently swirl the plate to mix and return to the incubator at 37°C and 5% CO_2.
36. Change the media completely with fresh NSC Differentiation media every 48 h for up to 14 days.
    a. Warm the NSC differentiation media to room temperature.
    b. Take an aliquot of 450 μL per well, and add 50 μL GANT58 or vehicle.

Note: NSC differentiation can be stopped at any time before 14 days, however for generation of mature MBP expressing oligodendrocytes, 14 days are recommended. Figure 4 shows the growth and change in morphology of the cells after plating.

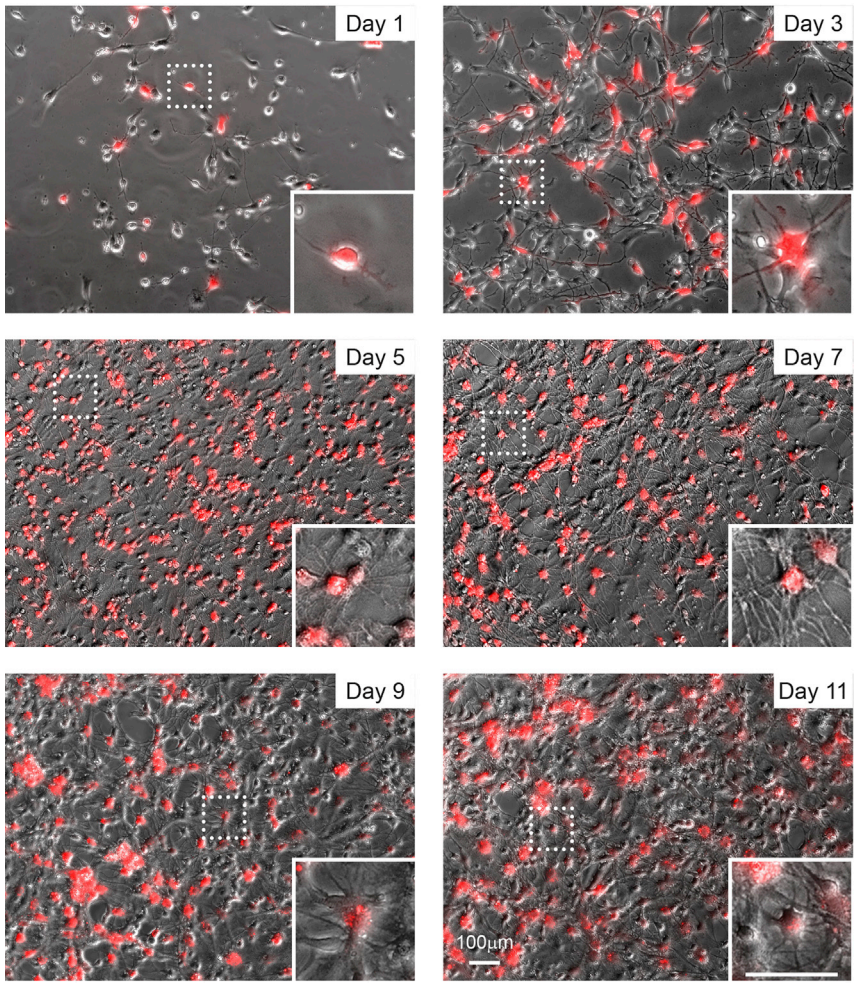

Figure 4. Differentiation of neural stem cells

Bright field images of neural stem cells (NSCs) superimposed on fluorescent images of RFP expressing NSCs after plating on GFR-Matrigel coated coverslips for differentiation. NSCs were imaged 1, 3, 5, 7, 9, and 11 days following plating. The cells continue to proliferate during the first 4–5 days of differentiation, and elaborate processes as they differentiate into the astrocytic, neuronal, and oligodendroglial lineage. Scale = 100 μm.

RNA extraction, proliferation assay, and immunofluorescent labeling

Timing: 2 h prep, 6 h for fixation and labeling

In these steps, RNA is extracted from the plated cells, and differentiated NSCs are treated with EdU and immunostained to examine their proliferation.

37. RNA extraction from adherent, differentiated neural stem cell cultures for downstream qRT-PCR analysis.
    a. Wash the cells twice with sterile HBSS or PBS.
    b. Add 250 μL of 0.25% Trypsin-EDTA to each well of a 24-well plate and incubate for 5–6 min at room temperature or until the cells begin to lift.
    c. Inactivate the trypsin by adding an equal volume of 1 mg/mL Trypsin Inhibitor solution to each well and transfer the cells from every 3 wells into a 2 mL microfuge tube.
    d. Centrifuge the cells at 500*g* for 5 min at 4°C.

e. Discard the supernatant and resuspend the pellet in 50 μL of Monarch DNA/RNA Protection Reagent (Monarch Total RNA Miniprep Kit).

Note: The cells can be stored at −80°C until RNA extraction using the Monarch Total RNA Miniprep Kit.

38. EdU labeling using Click-IT proliferation kit for the identification of proliferating cells.

Note: Follow the manufacturer's instructions in the Click-IT EdU Cell Proliferation Kit (C10340).

a. Prepare EdU stock solution: Dissolve 50 mg EdU in 9.9mL DMSO to make a 20 mM stock solution.
b. Dilute the 20 mM EdU stock solution to 20 μM in NSC differentiation media

Note: Mix 12 μL 20 mM EdU stock with 1.2 mL NSC differentiation media to make a 20 μM EdU working solution.

c. Add 50 μL of 20 μM EdU working solution to the cells in each well of a 24-well plate.
d. Incubate for 1 h.

39. Methanol fixation of differentiated neural stem cells for immunocytochemistry.
a. Pre-chill methanol (MeOH) to −20°C before beginning the succeeding wash and fixation steps.
b. Aspirate all the media from each well and wash with 500 μL 1× PBS.
c. Aspirate PBS from the wells and add 500 μL of chilled MeOH to each well.
d. Incubate the plates at room temperature for 10–15 min.
e. Aspirate the methanol from each well.
f. Wash each well for 3–5 min with 1× PBS.
g. Repeat the washes 3 times.

Note: Other fixatives like 4% Paraformaldehyde (PFA) and 10% Formalin can also be used. Cells can also be fixed with 4% PFA or 10% Formalin for 10 min before fixing with cold methanol. Cold methanol fixation works best for immunofluorescent labeling of MBP (myelin basic protein).

40. Remove each coverslip from its well using coverslip forceps and place it cell side up on a sheet of parafilm inside a humidified chamber consisting of a tray with moist paper towel.
41. Add enough 1× PBS to cover the coverslip (typically 100 μL or less) taking care not to drop the liquid in the center of the coverslip to prevent dislodging the cells. Troubleshooting 5

Note: For visualization of EdU, we use the Click-IT EdU Alexa 647 kit and follow the manufacturer's instructions. Calculate the total volume of EdU reaction solution needed based on the volume of liquid that covers each coverslip i.e. 100 μL per coverslip.

42. Immunofluorescent labeling
a. Wash coverslips with 1× PBS, 3 times for 5 min per wash.

Note: Immunofluorescent labeling can be combined with EdU labeling.

b. Block non-specific staining
i. Prepare fresh blocking solution consisting of 10% serum in the immunofluorescent labeling buffer solution consisting of 1% BSA and 0.25% Triton X-100 in PBS. The serum is from the same species used to raise the secondary antibody.

ii. 50 uL per 12 mm coverslip is sufficient to cover the cells.
iii. Incubate for 1 h at room temperature.
iv. Aspirate the blocking solution.

c. Dilute primary antibodies in the immunofluorescent labeling buffer solution.
d. Incubate the cells with primary antibodies for 3 h at room temperature.

Note: If needed, the cells can be incubated in primary antibody overnight (8–14 h) at 4°C.

e. Aspirate the primary antibody and wash 3 times with 1× PBS for 5 min per wash.
f. Dilute secondary antibodies in the immunofluorescent labeling buffer solution.
   i. All Invitrogen Alexafluor conjugated secondary antibodies, are used at a 1:1000 final dilution.
   ii. Add Hoechst (10 mg/mL stock) at a final dilution of 1:5000 (2 ug/mL final concentration) in the secondary antibody solution.
g. Aspirate the PBS and add 50–100 uL secondary antibody + Hoechst solution. Incubate for 1 h at room temperature.
h. Wash coverslips 3 times with PBS for 5 min per wash.
i. Using a P1000 pipette put 4 drops of Fluoromount-G onto a standard microscope slide.
j. Aspirate the PBS from the coverslips.
k. Slowly invert each coverslip on a drop of Fluoromount-G, total 4 coverslips per slide.
   i. Repeat this step as necessary for all coverslips.
l. Allow the Fluoromount-G to solidify overnight (12 h minimum, up to 24 h will not effect results) at room temperature before imaging.

EXPECTED OUTCOMES

One 100 mm plate of tertiary neurospheres typically generates ~1×10^7 total cells upon dissociation and more than 90% of the cells are viable after plating for differentiation.

When RNA is extracted after 14 days of differentiation, cells from 3 wells of a 24-well plate generate 600–1500 ng of total RNA at a concentration of 20–50 ng/mL.

Images of differentiated oligodendrocyte progenitor cells (OPCs), oligodendrocytes (OLs), astrocytes, and proliferating cells are shown in Figure 5. The exact percentage of cells generated depends on the pharmacological and genetic manipulations.

LIMITATIONS

This protocol works best for growing NSCs from the SVZ of 8–16 weeks old wildtype and multiple genetically modified C57bl/6 mice. We have also used this protocol for growing NSCs from 18 month old C57bl/6 wildtype mice but the NSCs grow very slowly, ~3 times slower than young adult NSCs, and require gentler handling.

TROUBLESHOOTING

Problem 1

No neurosphere growth from primary cells after 7 days

Neurospheres do not form following primary isolation and tissue dissociation (step 16).

Potential solution

Check growth factor concentrations and expiration dates. If all the reagents are appropriate, then decrease the incubation time of the dissected SVZ tissue with trypsin, from 5 to 3 min. Reduction of the concentration of trypsin from 0.25% to 0.05% can also help. We have observed efficient dissociation of NSCs without reducing cell survival by using either 0.05% Trypsin or papain for the entire

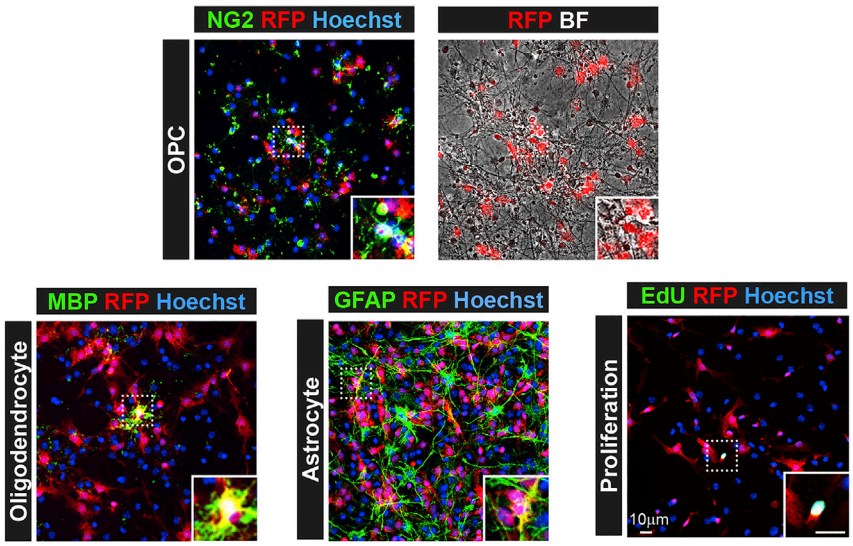

Figure 5. Immunofluorescent labeling of differentiated neural stem cells

Tertiary neurospheres were dissociated and plated on GFR-Matrigel coated coverslips in a 24-well plate for differentiation. Cells were immunolabeled after 14 days to detect co-expression of RFP in oligodendrocyte progenitor cells (OPCs) with NG2 antibody, oligodendrocytes with MBP antibody, astrocytes with GFAP antibody and proliferation with EdU labeling. BF = Brightfield, RFP = Red Fluorescent Protein, MBP = Myelin Basic Protein, GFAP = Glial Fibrillary Acidic Protein, Scale=10 μm.

experiment. Other laboratories have also used 0.025% Trypsin (He et al., 2009; Mich et al., 2014), and Accutase (Wachs et al., 2003) for dissociation with comparable cell survival.

Keep all the tools and brain slices on ice as much as possible. In addition, minimizing the total time from dissection to plating the primary cells is important to increase cell viability. Whenever possible, try to perform the dissection and dissociation steps quickly and efficiently, which may require multiple rounds of practice.

Problem 2

Failure of single cells to proliferate following passage of proliferative neurospheres.

This is most likely the result of extended incubation time in Trypsin during dissociation. Another reason could be over handling the cells by excessive pipetting or needle trituration during dissociation (step 23).

Potential solution

Decrease the incubation time and/or the concentration of trypsin, and gently pipette or triturate to dissociate proliferative neurospheres.

Problem 3

Proliferative neurospheres adhere to the plate

During the proliferative phase, neurospheres are adhering to the bottom of untreated culture plates in the absence of any adherent coating (Figure 3C; step 24).

Potential solution

In general, there will always be a low level of attachment to the plate, with some neurospheres completely adhering to the surface and beginning to differentiate. The attached neurospheres should be dislodged from the plate by gently pipetting the media over them at the time of

supplementing the media with growth factors. This will dislodge the most lightly attached spheres, and the remaining adherent neurospheres will differentiate. The differentiated cells remain firmly attached to the plate and are not carried over during further passaging.

Problem 4

Extensive cell death following nucleofection

Extremely low (<20%) cell survival following nucleofection (step 32).

Potential solution

Reduce the amount of time cells are in contact with the Mouse Nucleofector Solution. Typically, reducing the number of samples that are to be run in parallel is the simplest way to reduce the time. Working with one nucleofection reaction at a time is highly recommended to ensure the shortest time from resuspension to addition of NSC Proliferation media. After counting the cells, NSCs can remain as a suspension in the NSC Proliferation media, on ice, for up to 1hr without adverse downstream effects. Thus, aliquots of cells can be nucleofected and resuspended in the NSC Proliferation media sequentially, until the experiment is complete.

Problem 5

Loss of cells during immunolabeling

Extremely low number of cells on the coverslip following immunolabeling (step 41).

Potential solution

Hold the edge of the coverslip with forceps while transferring them to the parafilm taking care not to scratch the surface of the coverslip. Pipet the reagents gently on the edge of the coverslip. Do not squirt fluid in the center of the coverslip. Gently aspirate the fluid from the edge of the coverslip by pipetting. If a vacuum line is used for aspiration, cover the suction tube with a pipet tip to reduce the vacuum pressure.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jayshree Samanta (Jayshree.samanta@wisc.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate new unique code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

D.Z.R. and J.S. conceived of the experiments and framework. D.Z.R. performed the SVZ dissection, cell culture, immunofluorescent labeling, and analysis. D.Z.R. and J.S. wrote and edited the manuscript.

DECLARATION OF INTERESTS

A patent on the method of targeting GLI1 as a strategy to promote remyelination has been awarded, with J.L. Salzer, J. Samanta, and G. Fishell listed as co-inventors.

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