Video Article

Isolation and Expansion of Neurospheres from Postnatal (P1−3) Mouse Neurogenic Niches

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Abstract

The neurosphere assay is an extremely useful in vitro technique for studying the inherent properties of neural stem/progenitor cells (NSPCs) including proliferation, self-renewal and multipotency. In the postnatal and adult brain, NSPCs are mainly present in two neurogenic niches: the subventricular zone (SVZ) lining the lateral ventricles and the subgranular zone of the hippocampal dentate gyrus (DG). The isolation of the neurogenic niches from postnatal brain allows obtaining a higher amount of NSPCs in culture with a consequent advantage of higher yields. The close contact between cells within each neurosphere creates a microenvironment that may resemble neurogenic niches. Here, we describe, in detail, how to generate SVZ- and DG-derived neurosphere cultures from 1−3-day-old (P1−3) mice, as well as passaging, for neurosphere expansion. This is an advantageous approach since the neurosphere assay allows a fast generation of NSPC clones (6−12 days) and contributes to a significant reduction in the number of animal usage. By plating neurospheres in differentiative conditions, we can obtain a pseudomonolayer of cells composed of NSPCs and differentiated cells of different neural lineages (neurons, astrocytes and oligodendrocytes) allowing the study of the actions of intrinsic or extrinsic factors on NSPC proliferation, differentiation, cell survival and neuritogenesis.

Video Link

The video component of this article can be found at https://www.jove.com/video/60822/

Introduction

The neurosphere assay (NSA) was firstly described in 1992[1,2] and still remains a unique and powerful tool in neural stem cell (NSC) research. The isolation of NSCs from the main neurogenic regions has challenging issues because the requirements to maintain these cells in physiological conditions remain poorly understood. In the NSA, cells are cultured in a chemically defined serum-free medium with the presence of growth factors including the epidermal growth factor (EGF) and the basic fibroblast growth factor (bFGF)[1,2,3]. Neural precursor cells (stem and progenitors) are selected by using these mitogens since these cells are EGF and FGF-responsive entering a period of active proliferation while other cells, namely differentiated cells, die[4]. Neural precursor cells grow as neurospheres, which can be then passaged to further expand the pool of these cells[5]. Importantly, since these neural stem progenitor cells (NSPCs) are multipotent they are able to differentiate into the three major cell types of the central nervous system (CNS): neurons, oligodendrocytes and astrocytes[5].

The NSA provides a renewable source of undifferentiated CNS precursors, which can be used to study several processes including NSC proliferation and self-renewal, and neuronal and glial differentiation, in both physiologic and disease context. Moreover, in vitro studies can be used to evaluate the degree of intrinsic specification present in neural precursors during development, as well as to study the full potential of the cells, by removing extrinsic cues associated with their normal environment[6]. The neurosphere model is valuable to evaluate putative regulators since by maintaining the cells in a medium devoid of serum, the environmental cues are only provided by the surrounding cells[6]. Moreover, in the NSA, NSPCs are easily expanded in culture, the density of cells per area is high and the heterogeneous composition of the neurospheres has some similarity to in vivo niches[6]. These well-established advantages are the reason why this methodology has been widely used by many researchers.

The following protocol describes in detail all the processes from the isolation of postnatal NSPC population from the two main neurogenic regions, the subventricular zone (SVZ) and the hippocampal dentate gyrus (DG), to the expansion of those cells as neurospheres, as well as to the differentiation into neurons, astrocytes and oligodendrocytes. Lastly, different assays are also described to access stemness and multipotency properties of SVZ- and DG-derived NSPCs.

Protocol

All experiments were performed in accordance with the European Community (86/609/EEC; 2010/63/EU; 2012/707/EU) and Portuguese (DL 113/2013) legislation for the protection of animals used for scientific purposes. The protocol was approved by the "iMM's institutional Animal Welfare Body - ORBEA-iMM and the National competent authority - DGAV (Direcção Geral de Alimentação e Veterinária)."

1. Basic setup and preparation of culture medium

1. On the day of dissection, prepare the appropriate amount of growth medium corresponding to serum-free medium (SFM) composed of Dulbecco's modified eagle medium [(DMEM)/F12 with L-glutamine] (**Table of Materials**) supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin (pen/strep), 1% B27, with also 10 ng/mL EGF and 5 ng/mL bFGF. Warm the culture medium to 37 °C in a water bath.
   NOTE: The volume of growth medium depends on the number of pups, for 5 pups prepare ~100 mL (50 mL for SVZ and 50 mL for DG); however, after counting the number of cells (step 5.1) the exact volume will have to be adjusted.
2. For microdissection of SVZ and DG, prepare the calcium and magnesium-free Hanks' balanced saline solution (HBSS) dissection medium supplemented with 100 U/mL pen/strep.
   NOTE: Prepare 50−100 mL of dissection medium.
3. Set up a dissection microscope and prepare the tools needed to remove the brain (scissors and small spatula) and for SVZ and DG microdissections (Dumont small scissors, #7 forceps, #5 forceps, #5S forceps) by soaking in 70% ethanol.

2. Harvesting of postnatal (P1−3) mouse brains and SVZ/DG microdissections

1. Prepare 60 mm Petri dishes (growth area 21 cm^2) with HBSS supplemented with pen/strep and 2 sample tubes (one for SVZ and one for DG) with 500 µL of supplemented HBSS each.
2. Euthanize mice pups (P1−3) according to the protocol approved by the Institutional Animal Care facility/guidelines. Perform decapitation with a single incision with sharp scissors at the base of the brainstem.
3. Holding the ventral part of the body at the base of the head and using small pointed scissors, make a midline incision in the skin over the entire length of the head, thus revealing the surface of the skull.
4. Make a longitudinal incision at the base of the skull and continue cutting along the sagittal suture using small scissors with an angle shallow as possible in order to avoid damaging the brain structures.
5. Peel the skull to the sides using curved forceps and expose the brain.
   CAUTION: Make sure the dissecting instruments are free of ethanol before touching the brain.
6. Isolate the brain from the skull using a small spatula, by sliding under the base of the brain to cut the cranial nerves and blood vessels that are connected to the base of the brain, and transfer the brain into a Petri dish containing cold supplemented HBSS solution.
7. Place the Petri dish containing the brain under a dissecting microscope at low magnification and position the brain on its dorsal surface.
8. Using fine forceps, remove the meninges from the ventral side of the brain and the olfactory bulbs, while holding the brain in position by the cerebellum. Rotate the brain onto the ventral aspect and peel off the rest of the meninges.
   NOTE: Removing the dorsal meninges is a crucial step to ensure correct brain slicing.
9. Discard the cerebellum making a cut using forceps. Place a filter paper with a pore size of 11 µm onto a tissue chopper (**Table of Materials**) and set the brain onto the filter paper using curved-pointed forceps. Chop the brain into 450 µm coronal sections and use a wet lamina to collect the sectioned brain into a new Petri dish filled with cold supplemented HBSS.
10. To dissect out the SVZ, use forceps to separate coronal slices in an anterior-to-posterior fashion until reaching slices with the lateral ventricles, under a dissecting microscope.
11. Cut the thin layer of tissue surrounding the lateral wall of the ventricles (which corresponds to the SVZ) with fine forceps, excluding the striatal parenchyma and the corpus callosum. Isolate the SVZ by placing the tip of the forceps in the lateral corners of the lateral ventricle: one immediately under the corpus callosum and the other into the tissue immediately adjacent to the ventral area of the lateral ventricle. Then, cut a small line of tissue surrounding the lateral ventricle.
12. Collect the dissected tissue into a sample tube with supplemented HBSS solution previously identified as SVZ.
    NOTE: Exclude the SVZ in slices where both the lateral ventricles and the hippocampal formation begin to appear.
13. Go through all slices after SVZ microdissection in an anterior-to-posterior fashion and reach the hippocampal formation. Using forceps discard the first slice with hippocampus where DG is still unrecognizable.
14. To remove the DG, first isolate the hippocampus from the slices. Refocus the microscope to visualize the borders around DG.
15. Dissect the DG portion by performing a cut between the DG and CA1 region followed by a vertical cut between the DG and CA3 region using forceps. Remove fimbria and any adjacent tissue.
    NOTE: In P1−3 animals, the DG is almost undistinguishable from Ammon's horn but displays a small tip.
16. Collect the dissected tissue into a sample tube containing supplemented HBSS solutions previously identified as DG.
    NOTE: Overall injury to the hippocampus or surrounding area will make it more difficult to isolate the DG. Using an atlas of the postnatal mouse brain is essential when the user is not familiarized with the isolation of the SVZ and DG tissue from coronal sections.

3. Tissue dissociation

1. To dissociate the SVZ and DG tissue present in their respective tubes, add trypsin-EDTA 0.05% to have a final concentration of 5−10% of Trypsin-EDTA 0.05% in HBSS. Incubate for approximately 15 min at 37 °C, until the tissue is clumped together.
2. Wash the tissue from the trypsin by removing the media and adding 1 mL of new HBSS supplemented solution for 4 consecutive times.
3. Remove the HBSS and resuspend the digested tissue in 1 mL of SFM supplemented with 10 ng/mL EGF and 5 ng/mL bFGF. Mechanically dissociate the pellet by gently pipetting up and down approximately 7−10x using a P1000 pipette, until getting a homogenous cell solution.

CAUTION: Excessive mechanical dissociation can lead to increased cell death and will negatively impact subsequent cell growth.

4. Cell-pair assay to study cell fate

1. Prior to the experiment, prepare coated 24-well plates for adherent monolayer cultures according to sections 8-10.
2. To count the number of SVZ or DG cells (obtained in section 3) to be plated, use a solution containing 0.2% Trypan blue and count the cells using a hematocytometer.
3. Dilute the dissociated cell suspension in SFM supplemented with 5 ng/mL EGF and 2.5 ng/mL bFGF (low EGF/bFGF) at a density of 11,300 cells/cm^2 and plate them on coated glass coverslips.
4. After 24 h, fix the cells for immunocytochemistry against NSC markers such as sex determining region Y-box 2 (Sox2) and nestin as well as with a marker of the neuronal lineage (namely doublecortin [DCX], for immature neurons) (see section 14).
   NOTE: Sox2 is a marker of NSCs that undergo mitosis. $Sox2^{+/+}$ cell-pairs resulting from a single progenitor cell division reflects stem cell expansion[7].

5. Expansion of postnatal neural stem cells as neurospheres

1. To determine the density of the dissociated SVZ or DG cell suspension (obtained in section 3), count the cells using a hematocytometer.
2. Dilute SVZ and DG single cell suspension at a density of 2 x 10^4 cells/mL in SFM supplemented with 10 ng/mL EGF and 5 ng/mL bFGF. Seed SVZ and DG cells in uncoated 60 mm Petri dishes with a final volume of 5 mL/Petri dish.
3. Incubate SVZ and DG cells for 6−8 days and 10−12 days, respectively to form primary neurospheres, at 37 °C with 5% CO_2.
   NOTE: Incubation days more than those mentioned can promote aggregation of neurospheres and higher levels of cell death in the center of the neurosphere.
4. When the majority of neurospheres have a diameter of 150−200 µm, perform the neurosphere passage.
   NOTE: Passaging neurospheres when they do not have an appropriate diameter compromises all the next steps.

6. Passaging of neurospheres

NOTE: The following protocol can be applied to expand both SVZ and DG neurospheres.

1. To passage neurospheres, collect the SFM with growth factors containing neurospheres from the 60 mm Petri dish(es) and centrifuge for 5 min at 300 x *g*.
2. Discard the supernatant and resuspend the neurosphere pellet using a chemical dissociation kit (mouse) according to the manufacturer's instructions (**Table of Materials**).
   NOTE: Observe the incubation times precisely as they are crucial for performance.
3. Centrifuge for 5 min at 300 x *g*, remove the supernatant and add 1 mL of SFM supplemented with 10 ng/mL EGF and 5 ng/mL bFGF.
4. Triturate up and down approximately 10x with a P1000 pipette to dissociate neurospheres.
5. Count the number of cells using a solution containing 0.2% Trypan blue and a hematocytometer.
6. Reseed cells at a density of 2 x 10^4 cells/mL in uncoated 60 mm Petri dishes.
7. Incubate SVZ and DG cells for 6−8 days and 10−12 days, respectively to obtain secondary neurospheres, at 37 °C with 5% CO_2.
   NOTE: The self-renewal capacity of SVZ- and DG-derived NSPCs can be accessed by following protocol sections 5 and 6. For that, seed SVZ and DG cells at a density of 1.0 x 10^4 cells/mL (in uncoated 24-well plates) in growth SFM medium containing 5 ng/mL EGF and 2.5 ng/mL bFGF (low EGF/bFGF). Count the number of resulting primary and secondary neurospheres.

7. Storage of neurospheres

1. Collect the medium containing neurospheres (obtained from steps 5.3 and 6.7) from the 60 mm Petri dishes.
2. Centrifuge for 5 min at 300 x *g* and discard the supernatant.
3. Wash the cells 2x with 1 mL of HBSS (5 min at 300 x *g*).
4. Centrifuge for 5 min at 300 x *g*, discard the supernatant and store the pellet of neurospheres at -20 °C for molecular biology analysis.

8. PDL coating plate procedure

1. To prepare solution 1 (0.1 M borate buffer), weigh 3.92 g of boric acid and dilute in 400 mL of high purity water. Adjust the pH to 8.2 and make up to 500 mL with high purity water.
2. To prepare solution 2 (0.167 M borate buffer), weigh 10.3 g of boric acid and dilute in 900 mL of high purity water. Adjust the pH to 8.2 and make up to 1,000 mL with high purity water.
3. To reconstitute poli-D-lysine (PDL) (1 mg/mL in 0.1 M borate buffer), dilute 100 mg of PDL in 100 mL of solution 1.
4. Make aliquots of 10 mL to use immediately or freeze and store at -20 °C.
5. Under the laminar flow, add 1 coverslip per well and sterilize under UV light for 15 min.
6. Use the reconstituted PDL or thaw frozen reconstituted PDL.
7. Prepare the final solution of 100 µg/mL PDL in 0.167 M borate buffer by adding 10 mL of reconstituted PDL to 90 mL of solution 2.
8. Add the final solution to wells for a minimum of 2 h to overnight at 37 °C.
   NOTE: For 24-well plates, add a volume of 500 µL in each well.
9. Remove the solution and wash 3x with high purity water.
10. Let the coverslips dry in the laminar flow hood.
11. Leave the multi-well culture plates at 4 °C.

9. PDL/Laminin coating plate procedure

1. On day 1, coat the plates with PDL as described in section 8.
2. On day 2, remove the PDL solution and wash 3x with high purity water. Let dry.
3. Prepare 5 µg/mL laminin in cold SFM devoid of growth factors.
4. Add dissolved laminin to the coverslips and incubate at 37 °C overnight.
   NOTE: For 24-well plates, add a volume of 500 µL in each well.
5. Remove laminin using a pipette.
   NOTE: Do not wash the coverslips from laminin.
6. Use immediately or store at -20 °C.

10. Poly-L-ornithine (PLO) /laminin coating procedure

1. Under the laminar flow, add one coverslip per well and sterilize under UV light for 15 min.
2. Add 0.01% PLO solution to each well for 20 min at room temperature (RT).
   NOTE: For 24-well plates, add a volume of 500 µL in each well.
3. Remove the solution and wash 3x with sterilized 1x PBS. Let dry.
4. Prepare 5 µg/mL laminin in sterile 1x PBS.
5. Incubate for 2 h at 37 °C.
6. Remove laminin.
   NOTE: Do not wash the coverslips from laminin.
7. Use immediately.
   NOTE: Make sure that the coverslip is fully covered by the PLO solution by gently tapping the coverslip with a pipette tip. When shaken, the multi-well plates should make no sound.

11. Evaluation of neuritogenesis by generating a differentiated monolayer of cell

1. Collect media containing neurospheres from 60 mm Petri dishes (obtained from section 5) and centrifuge for 5 min at 300 x *g* at RT.
2. Discard the supernatant and dissociate the pellet of neurospheres in 1 mL of dissociation PBS (i.e., PBS without Mg^{2+}/Ca^{2+} and with EDTA [2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8.1 mM Na_2HPO_4 and 0.5 mM EDTA 4Na, at pH = 7.40]) by incubating for 15 min followed by mechanical dissociation. Alternatively, dissociate neurospheres using a chemical dissociation kit (mouse) (**Table of Materials**).
3. Centrifuge for 5 min at 300 x *g* at RT and discard the supernatant.
4. Resuspend the cell pellet in 1 mL of SFM devoid of growth factors.
5. Determine cell density using a hematocytometer.
6. Dilute the dissociated cell suspension in SFM devoid of growth factors at a density of 3,766 cells/cm^2 and plate cells on coated glass coverslips in 24-well plates.
7. After 1−3 days, fix cells for immunocytochemistry against a protein of the cytoskeleton (see section 14).

12. Differentiation of neurosphere cultures

NOTE: Neurospheres obtained from cell expansion, either from primary or passaged neurospheres (obtained in sections 5 or 6) can be differentiated into cells from different neural lineages.

1. When neurospheres have a diameter of 150−200 µm, collect 25 µL of neurosphere suspension medium and plate on coated glass coverslips, in 24-well plates.
   NOTE: To collect more neurospheres, gently rotate the Petri dish to concentrate the neurospheres in the center. Then, pipette from the center.
2. Put the plates in an incubator at 37 °C for 15 min so that neurospheres adhere to the substrate. Afterwards, add 500 µL of SFM devoid of growth factors (differentiative conditions).
3. After 24 h, replace the medium with fresh SFM devoid of growth factors.
4. Differentiate for different time points (e.g., 2 and 7 days in vitro, DIV2 and DIV7, respectively) with 5% CO_2 and 95% atmospheric air at 37 °C.
   NOTE: Cell survival, proliferation and differentiation can be analysed using different cell assays.

13. Cell biology assays

1. Cell survival assay
   1. Expose plated neurospheres to 3 µg/mL propidium iodide (PI) for 30 min before cell fixation in the incubator at 37 °C.
      NOTE: PI is an autofluorescent agent that is only able to enter cells with compromised membrane integrity[8]. Other methods to analyse cell survival can be used such as caspase 3 staining or the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay.

2. Cell proliferation assay
   1. Expose plated neurospheres to 10 µM 5-bromo-2'-deoxyuridine (BrdU) for 4 h before fixation in the incubator at 37 °C.
      NOTE: BrdU is a synthetic thymidine analogue that can be incorporated during DNA synthesis in proliferating cells[9].

3. Cell differentiation assay
   1. Expose 7-day-old plated neurospheres to 10 µM BrdU in the first 24 h, in the incubator at 37 °C.

2. Renew the SFM devoid of growth factors (differentiative conditions) and allow cells to develop in the absence of BrdU for the following 6 days until fixation.
   NOTE: These pulse-chase experiments, by co-labelling with markers of mature neural cells, allow the evaluation of progenitor cells that differentiate into mature cells during the protocol.

14. Immunostaining of neurosphere cultures

1. Cell fixation
   1. Prepare 4% paraformaldehyde (PFA) in 1x PBS and store at 4 °C or -20 °C.
   2. Remove the SFM devoid of growth factors from wells and add, to each well, 500 µL of 4% PFA at 4 °C for 20 min at RT.
   3. Wash 3x with 1x PBS, for 5 min each time, the coverslips containing differentiated neurospheres.
   4. Store coverslips until use in 500 µL of 1x PBS at 4 °C.
      NOTE: If the experiment does not have BrdU, skip to step 14.3.

2. Denaturation method (for BrdU experiments only)
   1. Prepare 1 M HCl at 37 °C.
   2. Rinse coverslips 3x in 1x PBS.
   3. Permeabilize cells for 30 min in PBS containing 1% nonionic surfactant (e.g., Triton X-100).
   4. Denature dsDNA with 1 M HCl pre-heated to 37 °C for 30−40 min at 37 °C (~300 µL/well).
   5. Wash wells 4x with 1x PBS.

3. Permeabilization and blocking
   1. Rinse coverslips in 1x PBS for 5 min.
   2. Incubate for 1.5 h with 0.5% nonionic surfactant and 3% bovine serum albumin (BSA) in 1x PBS (~300 µL/well).
      NOTE: For NeuN, use 6% BSA in 1x PBS.

4. Incubation and mounting
   1. On day 1, without washing, incubate cells with primary antibodies (**Table of Materials**) in 0.1% nonionic surfactant and 0.3% BSA in 1x PBS in the incubation chamber (for 24-well plates use 20 µL/well). Leave coverslips incubating overnight at 4 °C light protected if antibodies are conjugated to a fluorophore.
   2. On day 2, return coverslips to their respective wells and rinse 3x in 1x PBS for 5 min.
   3. Counterstain with appropriate fluorescence conjugated secondary antibodies (dilution 1:200) and with 12 µg/mL Hoechst 33342 in 1x PBS for 2 h at RT and light protected in the incubation chamber (20 µL/coverslip).
   4. Wash coverslips 3x in 1x PBS for 5 min.
   5. Mount coverslips onto microscope slides using 5 µL/coverslip of fluorescence mounting medium.
   6. Let coverslips air dry at RT, protected from light, for 1 day.

5. Microscopy
   1. View and acquire images using a fluorescence microscope.
   2. For each condition, use three replicates. Perform cell counts in five independent microscopic fields in each coverslip with a 40x objective (~100 cells per field).

15. Preparation of EGF and bFGF stock solutions

1. EGF stock solution
   1. To reconstitute lyophilized EGF, dilute the product in high purity water to reach a final concentration of 20 µg/mL.
   2. Aliquot and store in microtubes at -5 to -20 °C.

2. bFGF stock solution
   NOTE: bFGF must be reconstituted with a solution of 10 mM Tris, pH 7.6.
   1. Centrifuge the vial briefly before opening to bring the content to the bottom.
   2. Prepare 50 mL of 10 mM Tris, pH 7.6. For that, weigh 60.57 mg of Tris ($(HOCH_2)_3CNH_2$) and dilute it in 40 mL of high purity water. Adjust the pH to 7.6 and make up to 50 mL with high purity water.
   3. Prepare 10 mL of 0.1% BSA in 10 mM Tris, pH 7.6. For that, weigh 10 mg of BSA and dilute it in 10 mL of 10 mM Tris.
   4. Filter solutions prepared in steps 15.2.2 and 15.2.3 with a 0.22 µm filter under a laminar flow hood.
   5. Reconstitute 10 µg of bFGF in 1,000 µL of 0.1% BSA in 10 mM Tris with pH 7.6 to reach a final concentration of 10 µg/mL. Aliquot into microtubes at -20 °C for a maximum of 6 months.

Representative Results

SVZ and DG neurospheres, obtained by using the NSA, are composed of undifferentiated cells, positive for Sox2, a transcription factor involved in the self-renewal capacity and positive for nestin, an intermediate filament protein expressed in NSPCs (**Figure 1A**). In addition, SVZ-derived neurospheres have larger dimensions than their DG counterparts (**Figure 1A**). Importantly, in differentiative conditions, SVZ- and DG-derived NSPCs migrate out of neurospheres forming a pseudomonolayer of cells (**Figure 1B**).

To access the self-renewal capacity, the cell pair assay is performed based on the expression of Sox2 and nestin which tends to disappear in dividing cells that start the differentiation process with a combination of a marker of the neuronal lineage namely, DCX. In both neurogenic regions, it is possible to observe the presence of $Sox2^{+/+}$/$nestin^{+/+}$/$DCX^{-/-}$ symmetrical divisions (self-renewal) (**Figure 2A1,B1**), $Sox2^{-/+}$/$nestin^{-/+}$/$DCX^{+/-}$ asymmetrical divisions (**Figure 2A1,B2**) and $Sox2^{-/-}$/$nestin^{-/-}$/$DCX^{+/+}$ symmetrical divisions (differentiation) (**Figure 2A2,B1**).

Passaging the neurospheres increases the yield of NSPCs; however, cell death at DIV2 changes with passaging. In fact, the percentage of PI-positive cells is increased with cell passage in SVZ (P0: 15.6% ± 1.2% vs P1: 19.2% ± 2.7% vs P2: 32.35% ± 0.14% vs P3: 39.6% ± 4.0%) and in DG (P0: 16.31% ± 0.95% vs P1: 32.1% ± 1.7% vs P2: 27.42% vs P3: 32.2% ± 3.1%) (**Figure 3**).

Neuritogenesis can be evaluated in neurons obtained from the differentiation of SVZ and DG NSPCs at the beginning of differentiation: DIV1 (**Figure 4A,D**), DIV2 (**Figure 4B,E**) and DIV3 (**Figure 4C,F**). In fact, as observed in **Figure 4**, the length and ramification of the neurites increases with differentiation.

Cell proliferation can be evaluated in SVZ- and DG-derived neurospheres. Comparing primary differentiated neurospheres at DIV1 from SVZ (**Figure 5A1**) and DG (**Figure 5A2**), the percentage of BrdU-positive cells is higher in SVZ than in DG (SVZ: 6.15% ± 0.64% vs DG: 3.27% ± 0.13%; $p < 0.05$; $n = 4$; **Figure 5A3**). Moreover, cell differentiation can also be accessed by combining BrdU staining with a mature maker such as neuronal nuclei (NeuN) that identifies mature neurons (**Figure 5B1,B2**). **Figure 5B3** shows that the percentage of proliferating progenitors that differentiate into mature neurons is similar in SVZ and DG (SVZ: 12.04% ± 1.58% vs DG: 13.56% ± 0.48%; $p > 0.05$; $n = 4$).

The stemness and the multipotency of SVZ- and DG-derived NSPCs can be accessed using the NSA by evaluating the expression of different markers at different differentiation days (DIV2 and DIV7). Indeed, NSCs (nestin- and glial fibrillary acidic protein [GFAP]-double-positive cells) are present in both neurogenic regions (**Figure 6A,G**). These cells are able to differentiate into immature neurons (DCX-positive cells) (**Figure 6B,H**), mature neurons (NeuN-positive cells) (**Figure 6F,L**), oligodendrocyte precursor cells (neuron-glial antigen 2 [NG2] and platelet-derived growth factor receptor α [PDGFRα]- positive cells) (**Figure 6C,I**), mature oligodendrocytes (myelin basic protein [MBP]-positive cells) (**Figure 6E,K**) and astrocytes (GFAP-positive cells) (**Figure 6D,J**).

Different substrates can be used to coat coverslips to form the pseudomonolayer of cells under differentiative conditions. As shown in **Supplementary Figure 1**, DG cells migrate more when the coverslips have extra-coating with laminin combined with PLO or PDL than with PDL alone (**Supplementary Figure 1B−H**). In fact, when PDL and laminin are used together as substrates (**Supplementary Figure 1C,G**), DG cells form a more confluent pseudomonolayer than SVZ cells for which PDL is used alone (**Supplementary Figure 1A,E**).

Importantly, these results demonstrate the potential of the NSA to evaluate the stemness and multipotency properties of NSCs derived from the two main neurogenic niches.

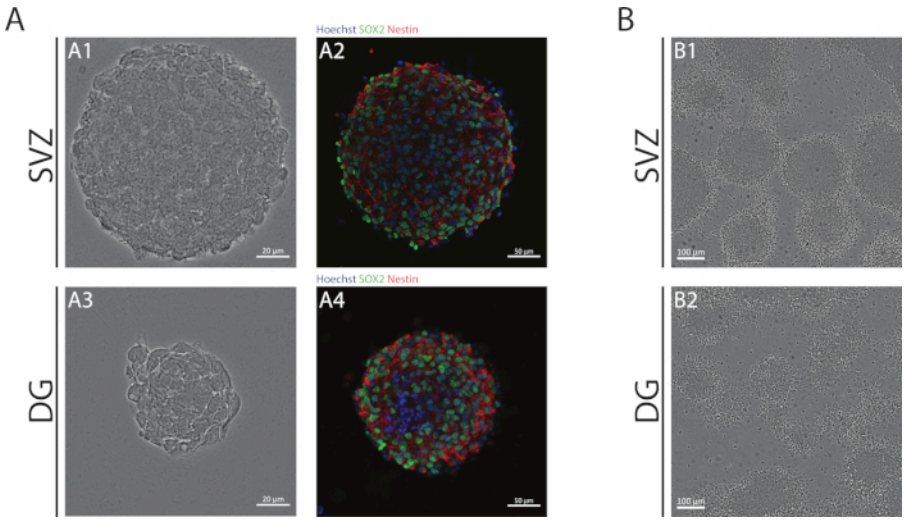


Figure 1: Subventricular zone and dentate gyrus derived NSPC cultured as neurospheres or as pseudomonolayers. (**A**) Representative brightfield (**A1,A3**) and fluorescence (**A2,A4**) images of SVZ- and DG-derived neurospheres, where nuclei were stained with Hoechst 33342 (blue) and NSCs for Sox2 (green) and nestin (red). (**B**) Representative brightfield images of pseudomonolayers generated from SVZ- and DG-derived neurospheres under differentiative conditions. Please click here to view a larger version of this figure.

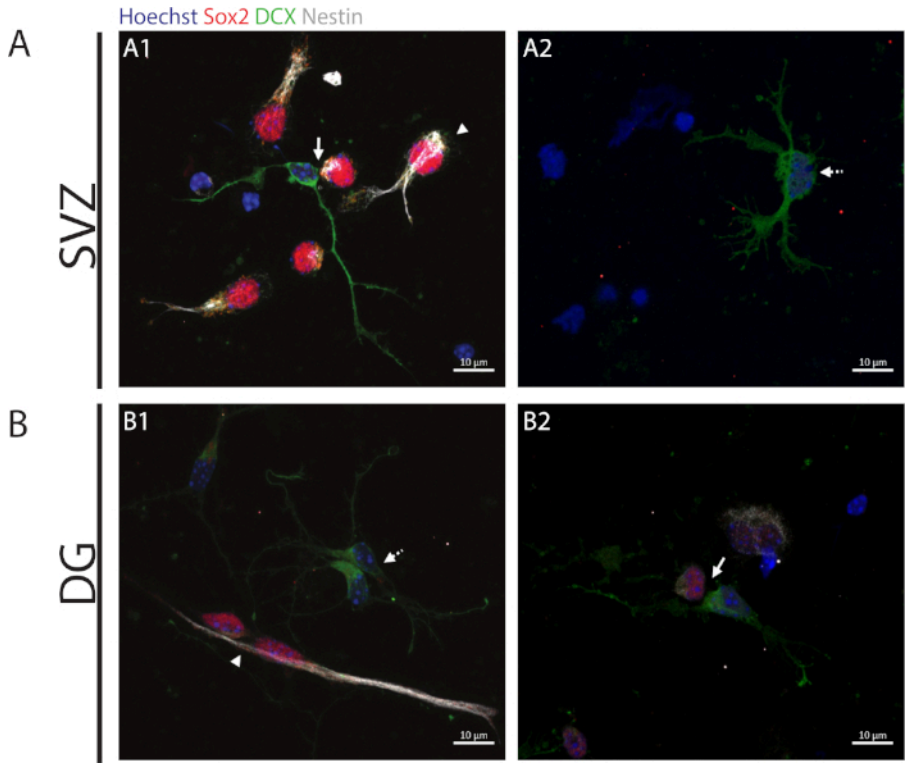


Figure 2: The cell pair assay. Representative fluorescence images of cell pairs derived from a progenitor cell division. SVZ and DG nuclei were stained with Hoechst 33342 (blue), stem-like cells for Sox2 (red) and nestin (white) as well as immature neurons with DCX (green). Arrowheads in panels A1 and B1 indicate $Sox2^{+/+}$/$nestin^{+/+}$/$DCX^{-/-}$ symmetrical self-renewing divisions, arrows in panels A1 and B2 indicate $Sox2^{+/-}$/$nestin^{+/-}$/$DCX^{-/+}$ asymmetrical divisions, dashed line arrows in panels A2 and B1 show $Sox2^{-/-}$/$nestin^{-/-}$/$DCX^{+/+}$ symmetrical differentiating divisions. Please click here to view a larger version of this figure.

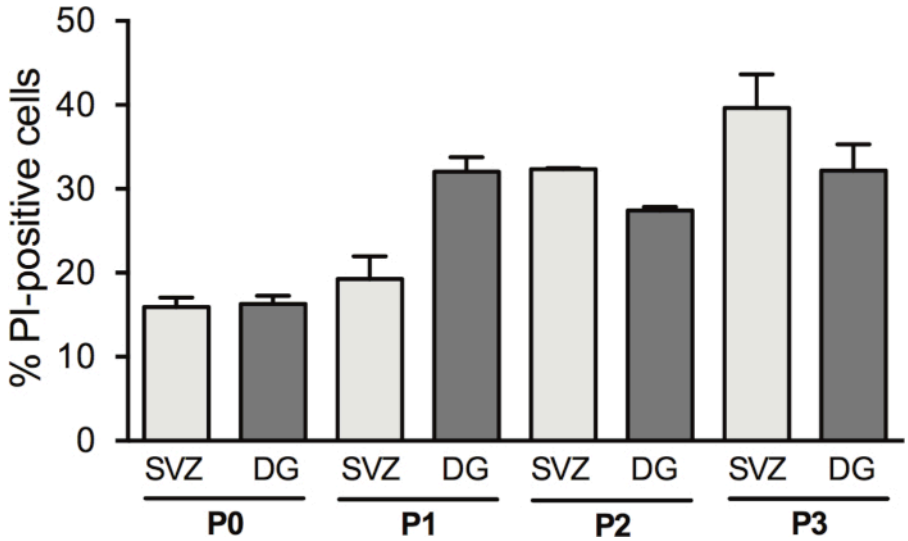


Figure 3: Cell survival analysis with cell passaging. Quantitative analysis of PI-positive cells at DIV2 in SVZ- and DG-derived differentiated neurosphere culture, after 0, 1, 2 and 3 passages (P0−P3). Data is expressed as mean ± SEM, n = 1−8. PI = propidium Iodide. Please click here to view a larger version of this figure.

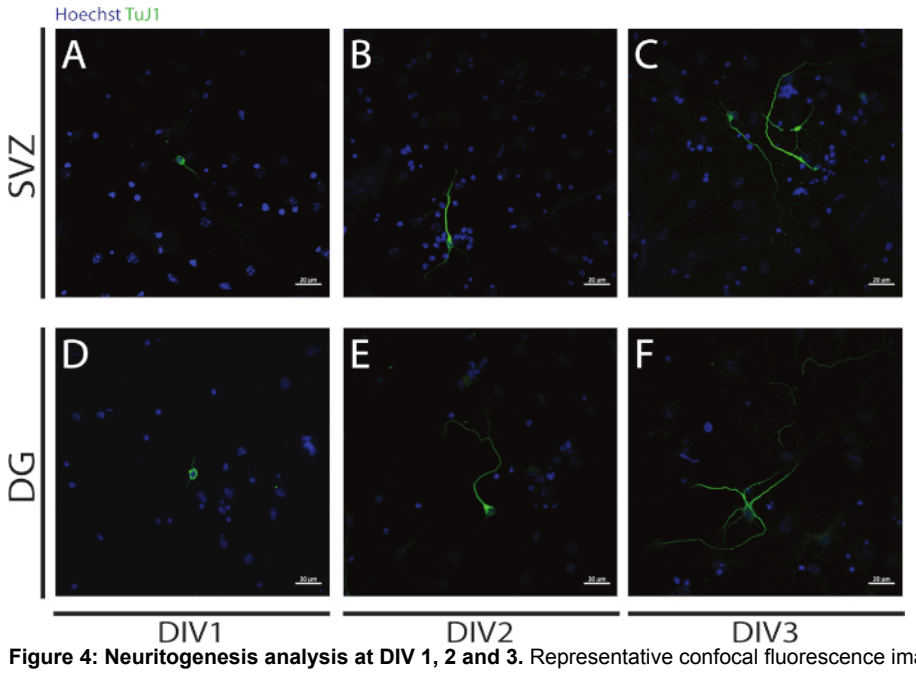


Figure 4: Neuritogenesis analysis at DIV 1, 2 and 3. Representative confocal fluorescence images of neurites, identified by the βIII-tubulin signal, in SVZ and DG neurons at (**A,D**) DIV1, (**B,E**) DIV2, and (**C,F**) DIV3. Please click here to view a larger version of this figure.

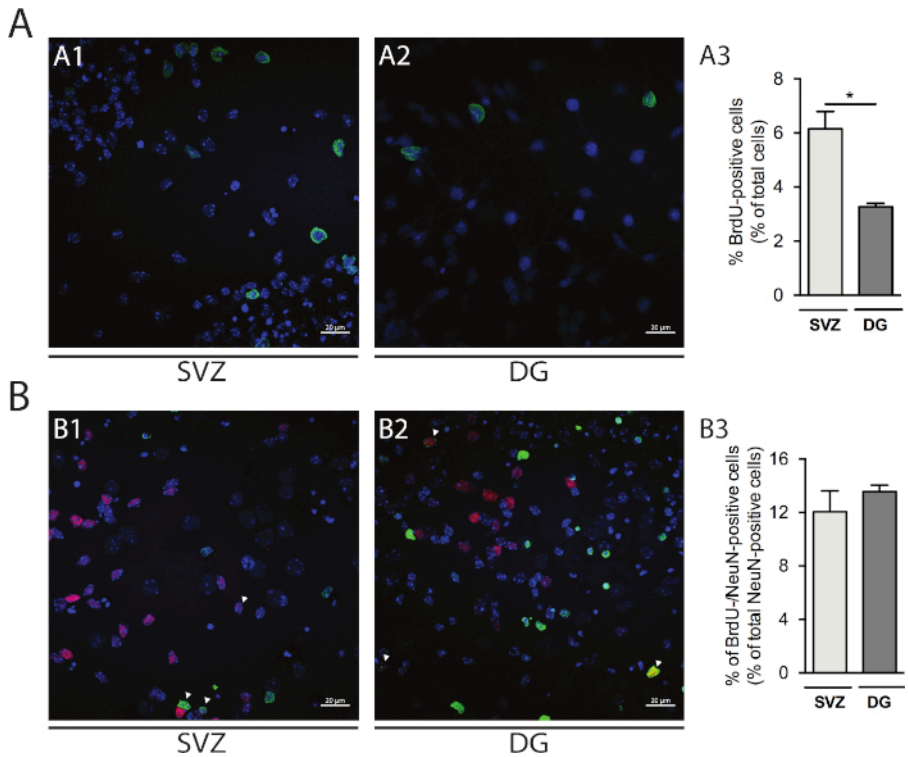


Figure 5: Cell proliferation assay. Representative confocal images of BrdU-positive cells at DIV1 in (**A1**) SVZ and (**A2**) DG. (**A3**) Quantitative analysis of BrdU-positive cells at DIV1 in DG- and SVZ-derived differentiated neurosphere culture. Data is expressed as mean ± SEM, n = 4. *p < 0.05 by t-test. Representative fluorescence images of BrdU- and NeuN-positive cells at DIV7 in (**B1**) SVZ and (**B2**) DG. Arrowheads indicate BrdU-/NeuN-positive cells. (**B3**) Quantitative analysis of BrdU-/NeuN-positive cells at DIV7 in both niches. Data is expressed as mean ± SEM, n = 4. BrdU: 5-bromo-2'-deoxyuridine, synthetic thymidine analogue. Please click here to view a larger version of this figure.

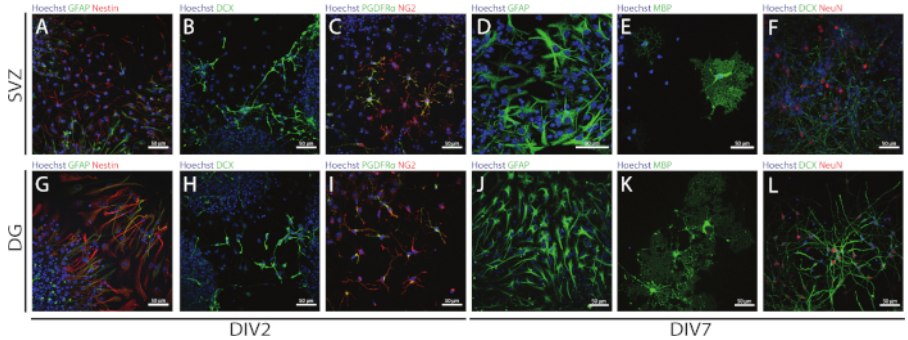
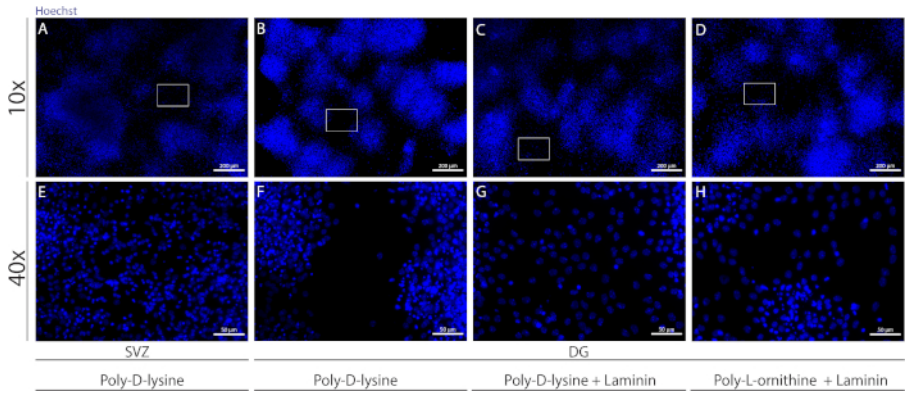


Figure 6: Neural cell types present in SVZ- and DG-derived differentiated neurosphere culture. Representative fluorescence images of SVZ- and DG-derived cell types after 2 and 7 days of neurosphere differentiation (DIV2 and DIV7), where cell nuclei were stained with Hoechst 33342 (blue) and: (**A,G**) NSCs for GFAP (green) and nestin (red), (**B,H**) immature neurons for DCX (green), (**C,I**) oligodendrocyte precursor cells for PDGFRα (green) and NG2 (red), (**D,J**) astrocytes for GFAP (green), (**E,K**) mature oligodendrocytes for MBP (green), and (**F,L**) mature neurons for NeuN (red). Please click here to view a larger version of this figure.



Supplementary Figure 1: Testing different substrates for neurosphere adherence and migration to form a pseudomonolayer. Representative fluorescence images of (**A,E**) SVZ-derived pseudomonolayer using poly-D-lysine as a substrate, (**B,F**) DG-derived pseudomonolayer using poly-D-lysine as a substrate, (**C,G**) DG-derived pseudomonolayer using poly-D-lysine with laminin as a substrate, and (**D,H**) DG-derived pseudomonolayer using poly-D-lysine with poly-L-ornithine as a substrate. Please click here to view a larger version of this figure.

Discussion

In vitro systems of NSPCs allow a better understanding of the cellular and molecular mechanisms, which can be further validated in vivo. The NSA is a very powerful method to mimic physiological conditions due to their three-dimensional structure. Moreover, this culture system is also technically easier to culture[10], compared with other in vitro systems such as the monolayer culture system. Indeed, with the NSA, it is easy to control the exposed extrinsic cues during cell development, either during the expansion or the differentiation phase, by adding precise and variable amounts of factors of interest to the media as well as by culturing neurospheres with other cell types[6]. Furthermore, compared with monolayer cultures, in the NSA, it is possible to obtain a higher cell density from a small amount of tissue or with a small number of cells, allowing parallel studies to be performed, thus reducing the number of animals[1].

The NSA is the most common method to isolate and expand NSCs[11,12,13], can be used to estimate the number of precursor cells present in a given tissue sample[5] and the precursor cell frequency between different conditions. However, both neurospheres and monolayer cultures do not account for quiescence NSCs[14]. Moreover, the NSA has some limitations[11,12,13] and the resulting neurosphere frequency depends on many factors including the medium components, the dissection procedure, the dissociation process[11,12,13], and neurosphere aggregation[5]. Indeed, in a high-density culture, neurospheres tend to aggregate. Consequently, caution must be taken when estimating the number of precursor cells in a sample. To overcome the above limitations, isolated NSPCs can also be expanded and passaged in a monolayer[5,15]. Importantly, using NSA to compare precursor cell frequency between different conditions is very useful and accurate because all these limitations are implicit and similar among all conditions performed in the same experiment.

There are critical steps in the neurosphere culture that need attention. In the brain harvesting step, complete removal of the meninges and good isolation of the neurogenic niches are essential to maximize the purity and yield of NSPCs. During tissue dissociation, due to the proteolytic activity of trypsin, excessive use of trypsin or longer incubation times can lead to cell lysis. Furthermore, the day of the passage is critical to obtain a healthy population of neurospheres. Passaging neurospheres with a diameter higher than 200 µm greatly affects the viability, proliferative and differentiative capacity of NSPCs. Importantly, longer cycles of passages, more than 10 can increase genetic instability[6]. Furthermore, coating with PDL and PLD/laminin for SVZ and DG cells, respectively, is essential to ensure good cell migration out of the neurospheres without compromising the differentiation process. In terms of the immunocytochemistry analysis, longer incubation times with PFA can compromise staining by masking the antigens and increasing the background.

The NSA is a powerful tool for providing a consistent and an unlimited source of NSPCs for in vitro studies of neural development and differentiation as well as for therapeutic purposes[16,17]. Indeed, this assay can be applied to genetic and behavioral models to further understand the molecular and cellular mechanisms involved in NSPC proliferation and differentiation[18,19]. This assay is also useful to test different drugs and compounds[20,21,22] as well as to perform genetic manipulations[19,23] to modulate NSC properties. In addition to immunocytochemistry, reverse transcription polymerase chain reaction and Western blot analysis can be performed to access RNA and protein expression, while electrophysiological studies and calcium imaging can be used to evaluate the function of the new-born neurons[21].

Disclosures

The authors have nothing to disclose.

Acknowledgments

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