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Primary Culture of Adult Neural Progenitors

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1. Introduction

In this chapter, we describe methodology for in vitro culture of adult neural stem and progenitor cells. The mammalian adult brain, once thought to be completely postmitotic, is now recognized to contain a finite number of neural stem cells, progenitor cells with the capacity for self-renewal and the ability to differentiate into functional neurons and glia *(1)*. The largest populations are found in the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus *(1)*. It is predicted that these populations, as well as smaller pools of stem and progenitor cells in other brain regions, will be affected by chronic exposure to drugs of abuse. In support of this hypothesis, psychomotor stimulants and opioids have been shown to influence activation of neural progenitors in adult tissue in vitro *(2)* and in vivo *(3)*. Toxic dopaminergic insult by repeated exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) increases progenitor proliferation and subsequent gliogenesis *(4)*. Chronic prenatal exposure to drugs of abuse such as cocaine impairs neurogenesis and migration of differentiating neurons *(5,6)*. One means of investigating the underlying molecular mechanisms responsible for these effects on neural precursors is through in vitro culture. In the protocol provided in the following subheadings, a methodology for the generation of primary stem and progenitor neurospheres from the subventricular zone of the C57Bl/6 mouse is described.

2. Materials

1. Male C57Bl/6 mice (n = 6) (Charles Rivers, St Foie, PQ), 8–10 wk of age.
2. Sodium pentobarbital.

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3. Dissecting microscope (Leica).
4. Artificial cerebrospinal fluid (aCSF): 26 m*M* $NaHCO_3$, 124 m*M* NaCl, 5 m*M* KCl, 2 m*M* $CaCl_2$, 1.3 m*M* $MgCl_2$, 10 m*M* D-glucose, 100 U/mL of penicillin, 100 µg/mL of streptomycin, pH 7.3.
5. Surgical instruments for microdissection (scalpel, dissecting scissors, and needle probes).
6. Dissociation media: aCSF containing high Mg^{2+} and low Ca^{2+} (26 m*M* $NaHCO_3$, 124 m*M* NaCl, 5 m*M* KCl, 0.1 m*M* $CaCl_2$, 3.2 m*M* $MgCl_2$, 10 m*M* D-glucose, 100 U/mL of penicillin, 100 µg/mL of streptomycin, pH 7.3). This solution can be aliquoted and frozen at –20°C. Add 0.2 mg/mL of kynurenic acid (Sigma) before use. Add 1.33 mg/mL of trypsin (Sigma) and 0.67 mg/mL of hyaluronidase (Sigma) before use.
7. Hybridization oven.
8. Polypropylene 15-mL and 50-mL capped tubes (VWR, Mississauga, ON).
9. 35-mm, 100-mm diameter, and 12-well tissue culture dishes with transwel inserts (VWR).
10. Enzyme inactivation media: Neurobasal media, 2 m*M* glutamine, 10% fetal calf serum, 10% horse serum, 100 U/mL of penicillin, 100 µg/mL of streptomycin (Invitrogen, Burlington, ON).
11. 30-mL syringe and 18-gauge needles.
12. Maintenance media: Neurobasal media, 2 m*M* glutamine, 25 mg/mL of insulin (Sigma), 100 mg/mL of transferrin (Sigma), 20 n*M* progesterone (Sigma), 60 µ*M* putrescine (Sigma), 30 m*M* sodium selenite (Sigma), 20 ng/mL of epidermal growth factor (Invitrogen), 10 ng/mL of basic fibroblast growth factor (Invitrogen).
13. Trypan blue stain (Invitrogen).
14. Orbital rotator/shaker.
15. Vortexer/mixer.
16. LabTek II Chamber Slide System (VWR).
17. Phosphate-buffered saline (PBS): 10 m*M* sodium phosphate, 154 m*M* NaCl.
18. Poly-D-lysine (1 mg/mL, Sigma) in double-distilled H_2O (ddH_2O). Dispense into 1-mL aliquots. Store at –20°C.
19. Poly-D-lysine/laminin coating solution (100 µg/mL of poly-D-lysine, 0.02 mg/mL of laminin). Add 1 mL poly-D-lysine stock (1 mg/mL) and 400 µL of 0.5 mg/mL of laminin (Invitrogen) to 8.6 mL of sterile ddH_2O. Use immediately.
20. Differentiation media with serum: Neurobasal media, 2 m*M* glutamine, 5% fetal calf serum, 5% horse serum.
21. Differentiation media without serum: Neurobasal media, 2 m*M* glutamine, 5 mg/mL of insulin (Sigma), 100 mg/mL of transferrin (Sigma), 20 n*M* progesterone (Sigma), 60 µ*M* putrescine (Sigma), 30 m*M* sodium selenite (Sigma), 1X B27 Supplement (Invitrogen).
22. Brain-derived neurotrophic factor (Invitrogen).
23. Matrigel (Becton Diskinson, Bedford, MA).

3. Methods

Methodology is described for tissue preparation (**Subheading 3.1.**), single cell dissociation/neurosphere culture (**Subheading 3.2.**), and assessment of progenitor differentiation potential (**Subheading 3.3.**).

3.1. Dissection and Tissue Preparation

This protocol describes the euthanasia procedure (**Subheading 3.1.1.**) and dissection parameters (**Subheading 3.1.2.**) underlying neurosphere preparation.

3.1.1. Animal Euthanasia

Mice were deeply anesthetized with sodium pentobarbital and decapitated. Brains were rapidly removed and blocked at approx bregma 1.4 and 0 mm under a dissecting microscope (*see* **Note 1**). Tissue blocks were placed in 10 mL ice-cold aCSF in 10-cm tissue culture dishes with gentle shaking on an orbital rotator until all blocks could be collected.

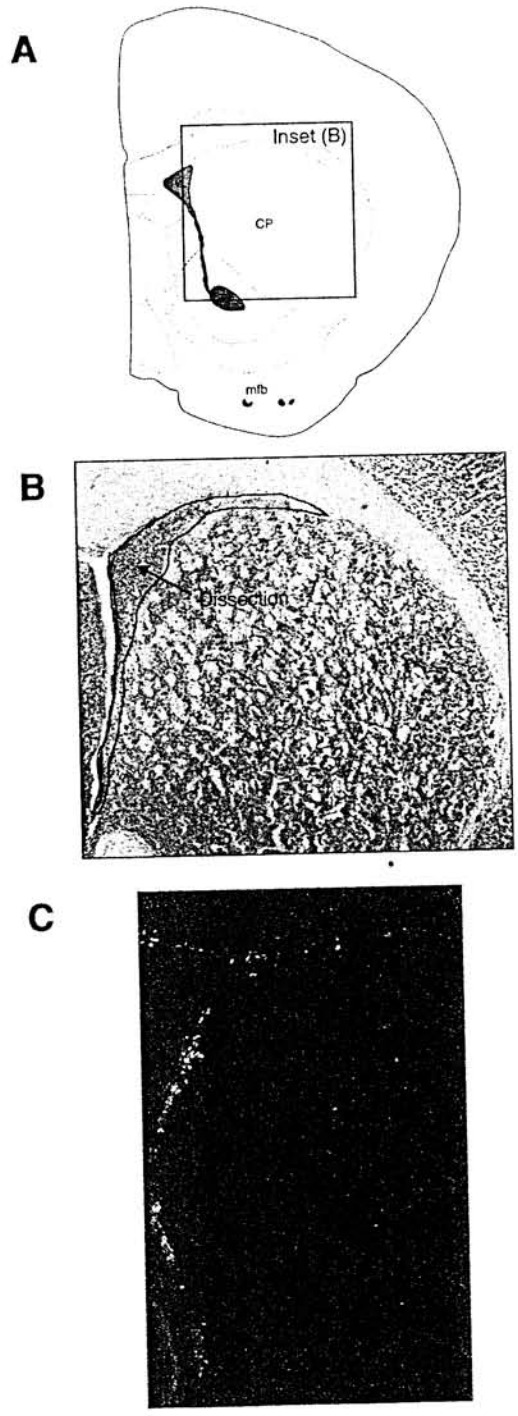
3.1.2. Tissue Dissection

Individual tissue blocks were transferred to 35-mm dishes, placed on their anterior face, and covered in ice-cold aCSF. The subventricular zone (approx 1.5 mm × 0.2 mm, L × W), including the ependyma and subependyma, was dissected from the striatal side of the lateral ventricles (**Fig. 1**) under a dissecting microscope using surgical instruments (scalpel, dissecting scissors, and needle probes).

3.2. Single-Cell Dissociation

Single cells were dissociated enzymatically in stepwise fashion.

1. Dissected tissue was minced into fine strips with a scalpel.
2. Minced tissue was transferred and pooled in a single 15-mL polypropylene tube.
3. Two milliliters of dissociation media was added and the tube was lightly vortex-mixed at the lowest vortexer/mixer setting. Tubes were capped and incubated for 30 min at 37°C in an hybridization oven with rotation.
4. Five milliliters of enzyme inactivation media was added and the sample was centrifuged for 5 min at 200*g*.
5. The supernatant was removed. Ten milliliters of enzyme inactivation media was added to the cell pellet and the suspension was transferred to a 50-mL polypropylene tube. Tissue was dissociated by tituration through successive 10-mL, 5-mL, 1-mL, and pasteur pipets.
6. The suspension was transferred to a 30-mL syringe and forced through an 18-gauge needle into a 50-mL polypropylene tube.


A
Inset (B)
CP
mfb
B
Dissection
C

7. The tube was centrifuged for 5 min at 200*g*.
8. The supernatant was removed and cells were resuspended in 10 mL of maintenance media.
9. Cells were counted on a hemocytometer using trypan blue. Twenty microliters of cell suspension was added to 20 µL of trypan blue solution (Invitrogen). Ten microliters of this cell/trypan blue mixture was counted on a hemocytometer. To progagate neurospheres successfully, approx 95% of all cells should exclude the dye after the dissociation procedure.
10. A total of 1×10^5 cells were plated per transwell well in 12-well tissue culture plate containing 3-µm pore transwell inserts (*see* **Note 2**). Cells were cultured in maintenance media. Cells capable of neurosphere formation were allowed to expand for 7–15 d (**Fig. 2**). Cultures were fed every 5 d.

3.3. In Vitro Assessment of Lineage Potential

Cell differentiation can be examined by plating neurospheres on tissue culture plates covered with extracellular matrix or other compound promoting neural cell adherence. Spontaneous differentiation potential into cells of neuronal and/or glial lineages can be assessed in serum containing media followed by immunocytochemistry. Neuronal differentiation can be facilitated by the omission of serum and the inclusion of brain-deprived neurotrophic factor (BDNF) in the culture media.

3.3.1. Coating Sterile Tissue-Culture Treated Microscope Slides

1. Tissue-culture treated glass LabTek II 4 -chamber slides were washed with 1 mL/well of PBS. PBS was removed by aspiration.

Fig. 1. *(see opposite page)* Dissection parameters. **(A)** Schematic diagram of a representative coronal section at bregma 0.9 in adult C57Bl/6 mice adapted from **ref. *10***. *Inset* B is boxed. **(B)** Cresyl violet-stained coronal section. The dissection area (subventricular, ventricular zone, and medial striatum) is outlined. **(C)** Increased magnification of the dissection area taken from an animal injected with bromodeoxyuridine (BrdU) to identify actively dividing cells. BrdU is a thymidine analog that readily passes the blood–brain barrier and is incorporated into the DNA of mitotic cells. BrdU (50 µg/Kg in sterile 10 m*M* PBS, pH 7.0) was administered intraperitoneally. Animals received two daily injections (4–5 h apart) over 2 consecutive days and a single injection on the third day. Mice were killed 24 h after the last injection and coronal sections processed for BrdU immunofluorescence. Note the presence of actively dividing cells in the dissection area. These cells have been shown to be a mixed population of neural stem and progenitor cells ***(1,8,11)*** that can be cultivated and expanded in vitro by neurosphere assay. VL, Lateral ventricle; CP, caudate putamen; mfb, medial forebrain bundle.

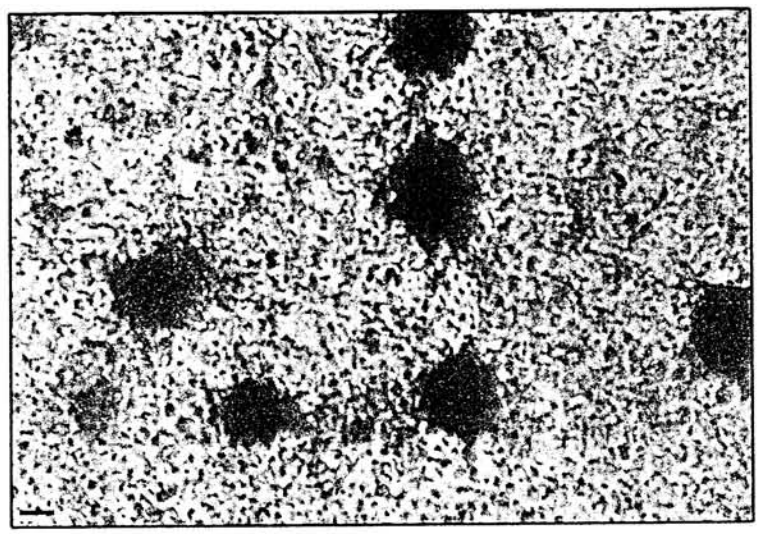


Fig. 2. Representative neurospheres. 7 DIV cultures photographed under bright-field illumination through the clear polyester transwell membrane on an inverted Leica microscope. Cellular debris and cells incapable of neurosphere formation can be seen among the neurosphere colonies. Scale bar, 50 µm.

2. Sufficient poly-D-lysine/laminin coating solution was added to cover each well (500 µL–1 mL) (*see* **Note 3**).
3. Wells were incubated at room temperature in a laminar flow hood for 1–2 h. Wells were covered with chamber lids to ensure that the coating solution did not dry. Slides were washed with PBS and used the same day (*see* **Note 4**).

3.3.2. Plating of Neurospheres

1. Neurospheres were gently removed from the transwell plates in a pasteur pipet.
2. Cells were gently dissociated by repeated pipetting through a 200-µL pipet tip.
3. Cells were plated in 1 mL of differentiation media with serum on poly-D-lysine/laminin-coated wells.
4. The following day, media was removed and cells were fed with 1 mL of differentiation media without serum. Cells were cultured for 20 d and fed every 5 d. Spontaneous differentiation to cells of neuronal or glial lineage can be assessed by monolayer culture in differentiation media containing 1% fetal calf serum followed by immunocytochemistry using antibodies raised against neuronal and glial lineage markers. Inclusion of 10 ng/mL of BDNF *(7)* in the differentiation media (in the absence of serum) can be used to promote selective differentiation of neurons.

4. Notes

1. Free-hand dissection can be imprecise. Precise anatomical alignment can be assured by sectioning whole brain into 500-µm slices on a vibratome as described in **ref. *8***. In this latter protocol, brains are removed, placed in 35-mm tissue culture dishes, and immediately covered with a thin layer of 1% Ultra-Pure low-melting point agarose (BRL) heated to between 38 and 40°C. Agarose-permeated tissue is placed at 4°C to allow the gel to harden rapidly. The agarose shell helps to maintain cerebral structure during sectioning *(9)*. Coronal sections (500 µm) are cut on vibratome in chilled aCSF, placed in 10-cm tissue culture dishes, covered in ice-cold aCSF, and processed as described in **Subheading 3.1.3.**
2. The number of cells plated per transwell will vary with the experimental design. To determine neurosphere yield per number of cells plated, total number of spheres per well are counted after 7–9 d in vitro (DIV). Uncoated 12- or 24-well culture plates can also be used but we have found that use make feeding of the neurosphere cultures easier for extended expansion. It has been observed by others that cells that do not divide and form neurospheres within 7 DIV are not subsequently activated by extended culture or by media replenishment *(8)*. Thus, if the experimental goal is to quantitate the number of colony forming units per cells plated, 7 DIV in uncoated tissue culture wells is sufficient. Larger neurospheres are obtained by longer culture times and repeated feeding. This expansion is necessary for experiments requiring large numbers of progenitors, although it should be cautioned that the effect of extended culture on neural stem and progenitor cell potential has yet to be established.
3. Plating efficiency and subsequent neuronal differentiation may be improved by coating cells with 1% fetal calf serum and Matrigel in lieu of poly-D-lysine/laminin coating solution *(8)*.
4. The coating solution can be reused immediately. To conserve costs, half of the total number of slides can be coated first. The coating solution can be removed and transferred to the remaining slides.

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