# Chapter 4

# Isolation of Adult Hippocampal Neural Progenitors

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## Abstract

Adult neurogenesis, or the creation of new neurons in adult organisms, is an exciting recent area of neurological research. The subgranular zone of the adult hippocampus is one area where hippocampal neural progenitors generate new neurons that functionally integrate into existing neuronal circuitry. Given the role that the hippocampus plays in learning and memory consolidation and its vulnerability to neurological diseases and conditions, such as Alzheimer's disease, understanding the mechanisms controlling the self-renewal and differentiation of neural progenitor cells is a critical first step in developing novel disease treatments. In this and subsequent chapters, we describe many of the *in vivo* and *in vitro* techniques necessary to study hippocampal progenitors in the adult rat. Specifically, this chapter details isolation of progenitors from the adult rat for the establishment of *in vitro* culture.

**Key words:** Neural, Hippocampus, Progenitors, Rat, *In vitro*, Adult, Isolation

## 1. Introduction

The discoveries that new neurons are generated continuously in some adult brain regions (adult neurogenesis) (1), and that the progenitor cells that generate these new neurons can be harvested and grown in culture (2, 3), have created exciting new possibilities for the treatment of many neurological diseases and injuries. *In vitro*, adult hippocampal neural progenitor cells (AHNPCs) can generate nearly all major cell types within the mammalian brain, including neurons, astrocytes, and oligodendrocytes, which makes them promising candidates for the treatment of neurological injuries and diseases. Additionally, neurogenesis may play roles in learning and memory (4–6), the effects of exercise on learning (6), stress and depression (7, 8), response to injury (9, 10), and aging (11). Therefore, understanding AHNPC behavior is important for developing the strategies needed to efficiently generate neurons,

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astrocytes, oligodendrocytes, or more AHNPCs for treatment of neurological disease or injury.

Here, we describe a protocol for the isolation of hippocampal progenitors from the adult rat brain, which is accomplished via a two-step cell fractionation process. Dissociated hippocampi from several rats are pooled and fractionated in a 35% Percoll solution. This first centrifugation separates progenitors and red blood cells (pellet) from other differentiated neural cell types (supernatant). The second fractionation step occurs in 65% Percoll and separates the red blood cells (pellet) from progenitors (supernatant). Once isolated, the progenitors are plated on poly-L-ornithine/laminin-coated culture dishes and can be repeatedly passaged. These cells have stable karyotype for up to 35 population doublings in culture (2) and can readily be cryo-preserved for long-term storage. See Chapter 5 for passaging and cryo-preservation protocols. Protocols for analysis of proliferation, immunostaining, and quantitative RT-PCR are also found in Chapter 5. Chapter 6 details *in vivo* engraftment and analysis, and protocols for viral production and transduction of AHNPCs are found in Chapter 7.

## 2. Materials

1. 3–6 female Fischer 344 rats (6-week-old; Charles River Laboratories, Wilmington, MA).
2. Medium Animal Guillotine (DCAP, 2.5″ opening, World Precision Instruments, Sarasota, FL).
3. Sterile scalpel blades (No. 10).
4. Dulbecco's Phosphate Buffered Saline (DPBS) w/o calcium/magnesium (Invitrogen, Bethesda, MD) + 1 g/L glucose. Sterilize through a 0.22 μm filter and chill on ice before use.
5. Papain, suspension (Worthington Biochemical, Lakewood, NJ). Store 4°C.
6. Deoxyribonuclease I, lyophilized (Worthington Biochemical). Store 4°C.
7. Dispase II (Roche Applied Science, Indianapolis, IN). Store 4°C.
8. Dulbecco's Modified Eagle Medium/Nutrient Mix F-12 (DMEM/F-12) with HEPES and L-Glutamine (Invitrogen).
9. Enzyme Cocktail: Dissolve 0.47 ml papain (2.5 U/ml), 19.98 mg deoxyribonuclease I (250 U/ml), and 400 mg Dispase II (1 U/ml) into 200 ml DMEM/F-12. Sterilize through a 0.22 μm filter, and freeze at −20°C in 50 ml aliquots. Warm to 37°C for approximately 30 min before use to fully activate the papain (see Note 1). Avoid freeze/thaw.

10. Percoll™ (Amersham, Piscataway, NJ). Store at 4°C.
11. 10× DPBS w/o calcium/magnesium (Invitrogen).
12. Percoll™ solution (100% Percoll™): Dilute 5 ml 10× DPBS into 45 ml Percoll™. Use immediately.
13. Fetal bovine serum (FBS, Invitrogen).
14. Harvest media: DMEM/F-12 containing 10% (v/v) FBS.
15. Trypan blue stain (Invitrogen).
16. Hemacytometer.
17. 3.5 cm poly-L-ornithine/laminin coated culture plate (see Subheading 2.2 for preparation).
18. DMEM/F-12 supplemented with 1% (v/v) N-2 Supplement (Invitrogen). Store supplemented medium at 4°C for up to 1 month.
19. Basic fibroblast growth factor (FGF-2, Peprotech, Rocky Hill, NJ).
20. FGF-2 stock solution: dissolve in DMEM/F-12 + N-2 at 0.1 mg/ml, aliquot, and store at −20°C. Once thawed, aliquot can be stored at 4°C for up to 1 week.

### 2.1. Anesthesia

1. Ketamine Hydrochloride, 100 mg/ml (Phoenix Scientific, St. Joseph, MO).
2. Xylazine Hydrochloride, 20 mg/ml (Ben Venue Laboratories, Bedford, OH).
3. 9% NaCl (w/v) in distilled/de-ionized water. Sterilize by 0.22 µm filtration.
4. Anesthetic cocktail: For each 150 g rat, sterilely mix 135 µl ketamine, 75 µl xylazine, 50 µl 9% NaCl, and 240 µl distilled/de-ionized water.

### 2.2. Laminin Coated Culture Plates

#### 2.2.1. Reagents

1. Sterile tissue culture water.
2. Sterile phosphate buffered saline (PBS), pH 7.4 (Invitrogen).
3. Poly-L-ornithine hydrobromide (Sigma, St. Louis, MO) dissolved in tissue culture water at 10 mg/ml. Sterilize by 0.22 µm syringe filtration, aliquot, and store at −20°C. Avoid repeated freeze/thaw.
4. Natural mouse laminin (Invitrogen). Store at −80°C. Thaw slowly at 4°C before use. Avoid repeated freeze/thaw and vortexing.
5. Plastic wrap.

#### 2.2.2. Preparation of Plates

1. Dilute poly-L-ornithine in tissue culture water to a final concentration of 10 µg/ml and add to tissue culture plates such that plates are well covered (~2 ml for 3.5-cm plate). Incubate overnight at room temperature. To maintain sterility,

leave the plates in a biological safety cabinet with blower off and sash closed. Leave UV light OFF.

2. Rinse plates twice with approximately the same amount of sterile water as used for poly-L-ornithine incubation. Do not allow plates to dry out.
3. Dilute laminin in sterile PBS to a final concentration of 5 µg/ml and add to plates at the same volume as was used for poly-L-ornithine.
4. Incubate at 37°C overnight.
5. Wrap in plastic wrap and store at –20°C for up to 6 months. Freeze plates on a level surface to avoid pooling and dry spots.

## 3. Methods

1. Anesthetize 3–6-week-old female Fischer 344 rats using ketamine/xylazine anesthetic cocktail (500 µL of anesthesia per 150 g of body weight) via intraperitoneal injection (see Note 2).
2. Wait several minutes to ensure that the rat is anesthetized. Check reflexes by pulling the hind limb laterally and pinching the hind paw while the animal is on its stomach. If the rat is not fully under it will retract its hind paw.
3. Decapitate the animals using the rodent guillotine, and as aseptically as possible, carefully remove the whole brain from the skull. Place brains in 50 mL centrifuge tubes containing 25 mL ice-cold DPBS w/o calcium/magnesium + 1 g/L glucose (see Note 3).
4. In a biological safety cabinet with autoclaved scalpels remove the hippocampi as aseptically as possible. Bisect each brain longitudinally and separate each hippocampal lobe from the cortical white matter using the natural separation along the alveus hippocampus. Remove as much white matter as possible (see Note 4).
5. In a sterile, dry tissue culture plate, finely mince the hippocampi using two sterile scalpel blades. Properly minced tissue should be easily aspirated into a sterile 10 ml pipette.
6. Add 3 ml enzyme cocktail per hippocampal lobe to 50 ml centrifuge tubes and warm in 37°C water bath for 30 min.
7. Transfer minced tissue into the warmed enzyme cocktail with a sterile 10 ml pipette. Incubate in 37°C water bath for 45 min with occasional gentle mixing. Tissue chunks will still be present, but the solution should be slightly turbid.

8. Centrifuge at 500 g for 5 min. Aspirate and discard enzyme cocktail supernatant.
9. Resuspend cells in 10 ml harvest medium and transfer to sterile 15 ml tube.
10. Centrifuge at 500 g for 5 min. Aspirate and discard supernatant.
11. Repeat Steps 9 and 10.
12. Resuspend cells with 10mL 35% Percoll solution in harvest medium. Thoroughly mix Percoll solution with cells and medium. Do not layer.
13. Centrifuge at 800 g for 10 min without brake. Progenitors are now in the pellet with red blood cells. Aspirate and discard supernatant.
14. Resuspend pellet with 10mL 65% Percoll solution in harvest medium.
15. Centrifuge at 800 g for 10 min without brake. Progenitors are now in suspension while the pellet primarily contains red blood cells.
16. Pipette supernatant into sterile 50 ml centrifuge tubes, noting the volume pipetted into each tube. Dilute the cell suspension with 4× volume of harvest media to decrease the buoyancy of the progenitors.
17. Centrifuge at 800 g for 10 min. Aspirate and discard supernatant.
18. Resuspend all pellets in a total of 10 ml harvest medium. Transfer to 15 ml centrifuge tube. Centrifuge at 800 g for 5 min. Carefully aspirate and discard supernatant.
19. Resuspend in 10 ml harvest medium.
20. Centrifuge at 800 g for 5 min. Aspirate and discard supernatant.
21. Repeat Steps 19 and 20. These pellets will be quite small and loose, so use care when aspirating supernatant to avoid disturbing the pellet.
22. Resuspend pellet in 2 ml DMEM/F-12 + N-2 and quantify cell concentration by hemacytometer using 1:1 DPBS/trypan blue solution to assess viability.
23. Thaw 3.5 cm poly-L-ornithine/laminin coated culture dish and aspirate PBS. Immediately plate well-mixed cells at approximately $10^5$ live cells per $cm^2$ (2 ml total volume). Do not allow the plate to dry out. Gently rock plate to evenly disperse cells. Do not swirl.
24. Supplement the culture with 20 ng/ml FGF-2 (0.2 µl of FGF-2 stock per ml of culture). Gently rock the plate to mix.
25. Incubate cells at 37°C in 5% $CO_2$ atmosphere.

26. Replace 50% of the medium with fresh, warm DMEM/F-12 + N-2 + 40 ng/ml FGF-2 every other day.
27. Cells should be ~80% confluent and ready to passage within 3–10 days. See Chapter 5 Subheading 3.1 for passaging protocol.

## 4. Notes

1. Papain is a cysteine-dependent protease. DMEM/F-12 contains the cysteine required for full papain activation.
2. Alternatively, $CO_2$ asphyxiation may be used for anesthetization followed by cervical dislocation.
3. If contamination is a concern, then 7.5 µg/ml of Fungizone® (Invitrogen) + 3%(v/v)penicillin–streptomycin(10,000U/ml, Invitrogen) may be added to the DPBS.
4. A dissecting microscope may be used for improved visualization of the appropriate brain structures during dissection.

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