Methods in Molecular Biology
DOI 10.1007/7651_2025_611

Evaluation of Adult Mouse Brain Neurogenic Niche Behavior Culturing Adult Mice Brain Slice In Vitro

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Abstract

Adult brain neural precursors carry out their biological activity in specific areas in which they are able to self-renew and differentiate into neurons. This is due to a complex microenvironment of cellular interrelations in which soluble factors from the neighboring cells, vascular structures, and the content of the brain ventricle cavity (cerebrospinal fluid) play a key role. This cellular functional entity, known as the "neurogenic niche," is able to generate new mature neurons, which are functionally integrated into the neuronal circuits of the adult mammal brain. The complexity of neurogenic niche signaling, *which include biologically active molecules* such as growth factors and morphogens, requires an experimental approach in order to create specific modifications of the biological activity of some of these molecules by means of a model of the active neurogenic niche, allowing an evaluation of neural precursor behavior.

Here we describe the adaptation of an in vitro culture technique of adult brain slices with selected coronal sections, involving the two main brain neurogenic niches, the sub-ventricular zone (SVZ), and the hippocampus dentate gyrus, together with their associated sub-ependymal zone (SEZ). We explain certain examples of the experimental approach to modify neurogenic niche soluble signaling, implanting latex microbeads as a carrier for soluble signals. Additionally, we introduce an immune-cytochemical approach involving bromodeoxyuridine detection as a neural precursor cellular lineage tracer in combination with different molecular expressions, as a means of testing progressive states of neural precursor differentiation and neuronal maturation.

This system represents a suitable strategy for evaluating the biological role of soluble components of the adult brain neurogenic niche.

Key words Brain neurogenic niche, Brain slices culture, Microbeads implant, Neural precursor, Neurogenesis

1 Introduction

Nowadays, the existence of neuronal replacement in the adult mammal brain is a fact. However, it seems to be a local process restricted to specific brain areas known as the "neurogenic niche." There are two consolidated neurogenic niches in the adult mammal brain—the sub-ventricular zone (SVZ) on the ventricular surface of the striatum, linked to olfactory bulb interneuron replacement, and the sub-granular zone (SGZ) of the hippocampal dentate gyrus,

linked to the sub-ependymal zone (SEZ) located on the ventricular surface [1, 2].

Neurogenic niches are described as complex biological structures around a cluster of neural stem cells termed "neural precursors"; *these derive ontogenically from the radial glia and have two main properties*—self-renewal and neuronal differentiation. A knowledge of the molecular mechanisms involved in activating the neurogenic niche is of great interest in the field of research, given their potential use as therapeutic replacement strategies in neurodegenerative diseases [3, 4].

Neurogenic niches are considered to be not only a cluster of neural precursors with self-renewing and neurogenesis properties but also a complex microenvironment with signaling interactions of neural precursors among them and other neighboring cells. This interaction is the result of the presence of soluble molecules such as growth factor and morphogens, which specifically regulate neural precursor behavior. Nowadays, many research studies aim to identify and monitor these neurogenic signals [5].

From the earliest stages of embryonic development to the adult stage, neurogenic niches are linked to the brain ventricles and their content, the cerebrospinal fluid. In fact, neural precursors are ontogenically located next to ventricular surface [6, 7].

Several previous research studies are focused on the composition and biological role of cerebrospinal fluid (CSF) during development and their differences in adult age [8]. In this regard, it has been proven that embryonic cerebrospinal fluid (E-CSF) contains biological components which have a significant bearing on the activation of neurogenesis in embryonic and adult brain neurogenic niches [9, 10].

We previously described a mesencephalic neuroepithelial in vitro culture technique, which demonstrated the involvement of embryonic-CSF in the early development of the neurogenic niche [11]. In this study, to test the neurogenic influence of embryonic-CSF on neurogenic niche activity in the adult mouse brain, we employed an in vitro culture technique with slices of adult mouse brain, that allowed us to identify and keep the SVZ and dentate gyrus neurogenic niches with their corresponding ventricular cavities accesible. At the same time, we were able to formulate experimental of loss or gain function strategies for specific soluble molecules during culture time. Finally, this technique also allows the development, with the cultured tissue, of histologic standard procedures of to evaluate with immunohistochemical approach induced changes in the neural precursor behavior [12].

2 Materials

2.1 Equipment

1. Biological Safety Cabinet class II.
2. Forced-air incubator at 37 °C and 5% CO_2.
3. Surgical pad for adult mouse.
4. Adult mouse brain matrix for guided gross sections.
5. Sterile dissecting tools (forceps, micro-scissors, micro-forceps, thin tungsten needle).
6. Vibratome LEICA (VT1200).
7. JP Selecta™ Precisdig Water Bath.
8. Stereo zoom microscope.
9. Steel grille.
10. Teflon rings of 2 mm high and 15 mm diameter.
11. Four wells in vitro culture plates, 15 mm diameter (NUNCLON).
12. PAP pen.
13. Confocal microscope (Leica TCS SPE).
14. Microtome (MICROM HM 330).
15. Microscope (Olympus).

2.2 Reagents and Materials

1. Carnoy's fluid: Prepare this fixative in a hood just before use. In 50 mL falcon tube, add 18 mL of ethanol, 3 ml of acetic acid glacial, and 9 mL of chloroform.
2. Tris–phosphate–BSA 1%: Add 1 g of albumin serum bovine to 100 mL of Tris–phosphate buffer, stir until clear, and, if necessary, heat the solution to 40 °C.
3. Phosphate buffer saline (PBS) 0.1 M: 19 mM sodium dihydrogen phosphate dihydrate and 80.73 mM disodium hydrogen phosphate (sodium dihydrogen phosphate dihydrate (2.97 g), disodium hydrogen phosphate (11.46 g), up to 1 litter of distilled water; adjust pH to 7.3).
4. DMEM supplemented culture medium with 1% Penicillin/Streptomycin (Gibco) + 25% of horse serum (Sigma) + 6 mg/mL Glucose +25% HBSS.
5. Anesthesia: 0.18 ml Imalgene® and 0.07 ml Rompun®.
6. 3% Liquid bacteriological agar for molecular biology (DIFCO Lab.)
7. Nitrocellulose membrane: Millipore filter paper (0.8 μm pore diameter).
8. Latex microbeads (50–100 μm diameter Sigma SD-91).
9. Sterile glass cover-slip.

10. Staining Dil: perclorato de 1,1′-dioctadecil-3,3,3′,3′--tetrametilindocarbocianina (“Dil”; DiIC18(3)-Molecular Probes Inc.).
11. Collagen Type I (Millipore) 1% in DMEM-Fetal Bovine Serum (Sigma) (8:1:1).
12. BrdU (10 μM); BrdU (3 g/l) (Sigma).
13. Anti BrdU primary antibody (1/50 dilution, Dako. Ref. M7240).
14. Anti Sox2 (D-17) primary antibody (1/50 dilution, Santa Cruz Biotechnology; Ref.sc-17319).
15. Anti β-III-Tubulin primary antibody (Tuj 1) (1/20 dilution SIGMA; Ref. T2200).
16. Anti Calretinin primary antibody (1:200 dilution, Millipore AB5054).
17. Anti Doublecortin primary antibody (DCX) (1/20 dilution, Abcam ab18723).
18. Secondary antibody Antigoat Ig G-Alexa 594 (1/1000 dilution Invitrogen; Ref.: A110 58).
19. Secondary antibody Antimouse Ig G-Alexa 488 (Invitrogen. Ref.: 10680), 1/1000 dilution.

3 Methods

3.1 In Vitro Culture of Adult Mouse Brain Sections

The aim of this technique was to develop an experimental approach that provided a direct convenient access to the two recognized neurogenic niches in the adult mouse brain—the sub-ventricular zone (SVZ) and the dentate gyrus of the hippocampus [13].

The in vitro culture technique with brain slices described makes it possible to introduce specific modifications in the neurogenic niche soluble signals involved in controlling neural precursor cells. This methodology also offers the possibility to monitor the behavior and evolution of neural precursor cells by means of a thymidine analog such as bromodeoxyuridine, a cellular lineage tracer under normal or experimental conditions.

Here, we set forth technical details to carry out the in vitro culture system with brain slices and explain certain experimental approaches to create specific changes in neurogenic niches. Finally, we propose a simplified immunohistochemical method to evaluate the degree of neural precursor differentiation and migration.

3.2 Obtaining Adult Mouse Brain Slices and the In Vitro Culture System

For this technique, we used adult mice of both sexes ranging from 3 to 6 months old, following the legal protocols for safeguarding and handling experimental animals, in accordance with the Institutional Care and Use Committee of the University of Valladolid.

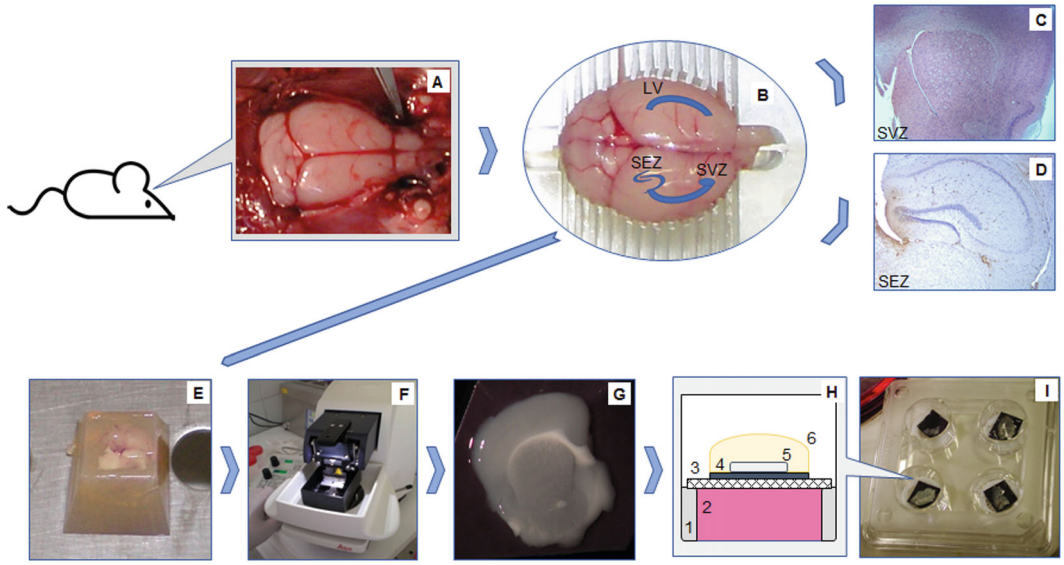

Fig. 1 Set of images showing organotypic tissue culture of adult mouse brain sections. (**a**) Opened skull and brain removal from adult mouse. (**b**) Adult mouse brain placed in a gross section guided matrix, showing lateral ventricle (VL) and neurogenic niches, subependymal zone (SEZ) and subventricular zone (SVZ) location. (**c**, **d**) Histological sections with Nissl stain showing SVZ and SEZ structure, respectively. (**e**) Mouse brain embedded in agarose ready to be sectioned with a vibratome (**f**). (**g**) Section from the SVZ obtained with a vibratome placed onto the filter membrane. (**h**) Diagram showing the disposition of culture system components: 1-Teflon ring. 2-Culture medium. 3-Steel grille. 4-Filter paper. 5-Brain section. 6-Colagen drop. (**i**) Image of the culture plate containing the brain sections over the filter membrane supported by the Teflon ring and the steel grille, in contact with the culture media, ready to be incubated

- Adult mice under deep anesthesia with intraperitoneal injection of ketamine and Rompun were sacrificed by cervical dislocation. The dorsal head skin was sterilized with 96° ethanol and a sagittal section was made with a scalpel to reveal the dorsal surface of the skull (*see* **Note 1**).
- The skin of the head was fixed to the surgical pad with thin needles, and with the use of surgical micro-scissors, we carefully made a medium sagittal section from the occipital to the frontal bones of the skull (*see* **Note 2**).
- Both sides of the opened skull were laterally displaced with surgical forceps until fracture, exposing the dorsal surfaces of the cerebellum, brain hemispheres, and olfactory bulbs (*see* Fig. 1).
- With a surgical micro-spoon, we carefully elevated both brain hemispheres and the cerebellum, which were liberated with micro-scissors (*see* **Note 3**).
- The brain hemispheres and cerebellum block were immediately immersed in 4 °C sterile saline solution and refrigerated for 1.5 h (*see* **Note 4**).

- Afterward, the pieces of the brain hemispheres and cerebellum block were placed and carefully positioned in a sterile adult mouse brain *matrix to guide gross sections* (*see* Fig. 1). Following this, the part of the brain hemispheres containing the SVZ or the dentate gyrus of the hippocampus was isolated (*see* **Note 5**).
- Each brain hemisphere block was placed and carefully positioned in 3% agarose fluid and left to solidify at room temperature for a pyramidal block to be carved.
- By means of an automatic vibratome, we obtained consecutive 300 μm coronal brain sections, which were placed on a culture plate with a culture medium. Under a binocular microscope, we selected the sections whose morphology was compatible with the neurogenic niche area (SVZ or dentate gyrus) for in vitro culture (*see* **Note 6**).

3.3 Brain Slices In Vitro Culture System

Coronal brain sections, in which the neurogenic niche (SVZ or dentate gyrus) and lateral brain ventricle were well preserved, were selected for in vitro culture.

- After sectioning, the brain slices remained immersed in the culture medium and a small square of Millipore filter paper was placed next to it. With the use of a micro-forceps, the brain sections were deposited on the dark side of the filter paper and both were put in a culture well.
- We used four sterile culture plates with four well of 15 mm in diameter. At the bottom of each well, there was a Teflon ring with a steel grill on the top, and the inner space of the Teflon ring was filled with 300 μL of culture medium making contact with the steel grill.
- Each brain section together with its filter paper support was deposited on top of the steel grill in direct contact with the culture media, in order to promote diffusion (*see* Fig. 1).
- Finally, to prevent the surface section from drying, we covered it with a drop of 1% collagen solution.
- In vitro culture was in a forced air incubator, at 37 °C and 5% CO_2, for 7 days, with the culture media changed every 2 days.
- After culture, the slides were fixed in Carnoy solution (*see* **Note 7**), dehydrated in graded alcohols, and embedded in paraffin following standard protocols. 8 μm-thin sections were obtained for histological and immunocytochemical studies.

3.4 Experimental Strategies Using Brain Sections in an In Vitro Culture System

- All the brain slice cultures were supplemented with a 10 μM concentration of BrdU in the culture medium. BrdU is a thymidine analog which is incorporated in the newly synthesized DNA and which can be used as a cellular tracer for neural precursor cell progeny, in combination with other molecular markers, to

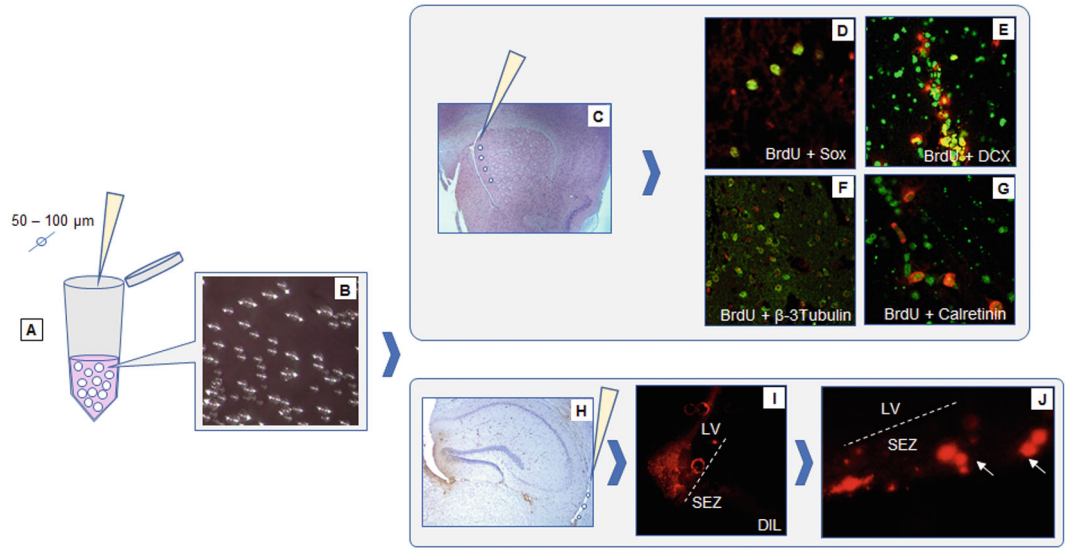

Fig. 2 Set of images showing experimental strategies associated with the in vitro culture system of brain slices. (**a**) Latex microbeads soaked in culture medium. (**b**) Latex microbeads on a filter paper ready for handling. Histological sections showing microbeads implanted (white circles) in the SVZ (**c**) or inside the lateral ventricle cavity (**h**). (**d**–**g**) Confocal photomicrographs of neural precursor immunolabeling showing different degrees of differentiation. Images show double immunolabeling with (**d**) anti-BrdU (green) and anti-Sox2 (red) antibody; (**e**) anti-BrdU (green) and anti-DCX (red) antibody; (**f**) anti-BrdU (green) and anti-β-3Tubulin (red) antibody; (**g**) anti-BrdU (green) and anti-Calretinin (red) antibody. (**i**) DIL-labeled microbeads implanted inside the lateral ventricle close to the SEZ. (**j**) Arrows show migratory neural precursors in the SEZ after DIL labeling

detect cellular migration and differentiation (*see* **Note 9** and Fig. 2).

- Specific experimental conditions can be achieved by addition to the culture medium of biologically active molecules such as growth factors, morphogens, or others, including complex biological fluids, for instance, cerebrospinal fluid [14]. The strategy proposed in the previous paragraph makes it possible to study their influence on the behavior of neural precursors.
- In order to incorporate local changes in neurogenic niche surroundings, we used synthetic latex microbeads previously soaked in a solution of biologically active molecules (for instance, E-CSF, growth factors such as FGF-2, or morphogens such as retinoic acid). We also employed soaked microbeads to administer fluorogenic lipophilic markers (DIL). Microbeads were surgically implanted in the brain slice at the beginning of the culture (*see* **Note 8**), in both the brain striatal parenchyma in the SVZ and the brain ventricle cavity [15] (Fig. 2).
- From the microbeads, the soaked molecules created a diffusion gradient capable of locally influencing the neurogenic niche or, in case of fluorogenic lipophilic dyes (DIL), of permitting

incorporation in the neural precursor cellular membrane, making it possible to monitor cellular migration by laser confocal microscopy during the brain slice culture period.

3.5 Protocols for Immunocytochemical Evaluation of Neural Precursor Behavior

As said before, all the cultured brain slices were exposed to BrdU (thymidine analog), with incorporation in the DNA of the newborn neural precursors in the neurogenic niche and detectability remaining over a long period. This was very useful as a neural progenitor cellular lineage tracer. Consequently, our immunocytochemical proposal is based on double cellular colabeling, including BrdU with other molecular markers for the state of neural precursor differentiation, Sox2 (an undifferentiated state marker); Doublecortin, (a specific marker for migratory precursors); βIII-Tubulin (a newborn neuronal marker); and Calretinin, a differentiated neuronal marker [16].

The source of the primary and secondary antibodies is described in the Reagents and Materials chapter, and the immunolabeling procedures were standard, following the manufacturer's instructions. Immunolabeled cells were photographed with a Leica TCS SPE confocal laser microscope.

4 Notes

1. All the procedures for in vitro culture of brain slices were conducted in a biological safety cabinet under sterile conditions.
2. To avoid brain tissue damage, the tip of the micro-scissors must be next to the inner surface of the skull bones, and the cut line must follow the brain's interhemispheric fissure.
3. Isolation of the brain's hemispheres and cerebellum must be carried out very carefully to avoid neural tissue damage. First, we sectioned the olfactory bulbs, and with a micro-spoon, the ventral surfaces of the brain hemispheres were elevated alternatively; then, with the use of micro-scissors, the mesencephalon was sectioned, and we could elevate the brain and cerebellum block on the top of the micro-spoon.
4. This is a usual procedure for depressing cellular metabolism and minimizing tissue damage during sectioning.
5. The mouse brain matrix allowed us to make coronal sections with a separation of 1 mm. With surgical razor blades, we separated both hemispheres and discarded the anterior and posterior poles of each one.
6. To recover the brain slices, we made a small culture media pool over the vibratome razor blade in which each section remained

floating, recovery being performed with a sterile glass cover slip for transfer to the culture media dishes.

7. Fixing was initially conducted by addition of Carnoy's fixative drops *over* the brain slice and the filter paper together to preserve surface flatness. This is appropriate in order to obtain 8 μm histological sections from 300 μm brain slices.
8. Regarding the microbeads, after 2–3 h of soaking at 4 °C, they were aspirated with a micropipette and layered on top of a wet filter paper. With the tip of a thin forceps, each microbead was carefully transferred and inserted in the nerve tissue, or alternatively in the brain ventricle cavity in the section.
9. Alternatively, BrdU administration can be performed by intraperitoneal injection of two 0.1 mL. single doses of BrDU 3 g/l, 7 days before the mice are sacrificed [17].

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