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# *In vitro* isolation of neural precursor cells from the adult pig subventricular zone

Olivier Liard[a,1], Stéphanie Segura[b,1], Aurélie Pascual[c], Pierrette Gaudreau[d], Thierry Fusai[c], Emmanuel Moyse[b,d,*]

[a] *Service de Chirurgie Maxillo-faciale et Plastique de la face, HIA Laveran, BP 50, 13998 Marseille Armées, France*
[b] *Physiologie Neurovégétative, UMR 6231 CNRS, Université Aix-Marseille-3, Case 351, Avenue Escadrille Normandie-Niemen, F-13397 Marseille cedex 20, France*
[c] *Unité de Chirurgie et Physiologie Expérimentales (UCPE), Institut de Médecine Tropicale, 58 Boulevard Charles Livon, 13007 Marseille, France*
[d] *Neuroendocrinologie du Vieillissement, Centre Hospitalier de l'Université de Montréal, Biotechnologies Angus, 2901 Rue Rachel Est, Montréal, QC, Canada, H1W4A4*

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ABSTRACT

In order to improve cell therapy techniques, we have characterized a multipotent neural precursor cell isolation technique from the subventricular zone of adult pig brain. The pig is a non-primate species that is immunologically closest to human. The proliferative zone of this neurogenic structure was first localized *in situ* in the pig brain by Ki-67 immunohistochemistry, as a ventral subfield of the Nissl-stained subventricular zone. For *in vitro* cultures, the striatal forebrain was sampled from deeply anaesthetized adult pigs and SVZ tissue explants were immediately microdissected out and dissociated in the appropriate medium. Primary cell culture in the presence of EGF and bFGF allowed growth of spherical masses that exhibited sustained growth and self-renewal capacity through six subsequent passages. Molecular characterization using reverse transcription-polymerase chain reaction (RT-PCR) showed that expanded pro-differentiating neurospheres expressed markers of proliferation, neural stem cells, and committed neural progenitors. After growth factor removal, the spheres became adherent and were shown to contain the three neural cell lineages by triple immunocytofluorescence and confocal microscopy. The present protocol therefore allowed for *in vitro* expansion of pig brain primary cells that display capacities for proliferation, self-renewal, and multipotency, i.e., the cardinal features of multipotent neural precursor cells.


## 1. Introduction

Neural stem cells are identified in primary cultures of adult nerve tissue treated with EGF and bFGF by the growth of cellular masses called "neurospheres" which include three neural lineages: neurons, astrocytes, oligodendrocytes (Reynolds and Weiss, 1992; Weiss et al., 1996). Their discovery in adult mammals has suggested great promise for clinical repair in neurology (Lindvall and Kokaia, 2006). A basic limitation upstream of purported neurological applications is the use of rodent cell cultures for study, since even *in vitro* neurosphere assay conditions are suspected to vary across species (Reynolds and Rietze, 2005; Ray and Gage, 2006). Regulatory mechanisms should therefore be investigated in neural stem cells from adult humans, or at least, from the most closely related non-primate animal species, which is the pig (Bucher et al., 2005; Vodicka et al., 2005). Pig neural stem cells have only been isolated from the foetal brain to date (Schwartz et al., 2005; Harrower et al., 2006). However, extracellular signal competence is known to undergo drastic modifications throughout ontogeny (Wada et al., 2006) and foetal stem cells have proven to be more tumorigenic than adult stem cells (Suzuki et al., 2005; Foroni et al., 2007; Shih et al., 2007). To overcome these problems, we developed experimental procedures that allow successful primary culturing of neural stem cells from the adult pig. In order to minimize the delay between animal sacrifice and tissue sampling, especially because of skull thickness, we performed the surgical collection of brain tissue in deeply anaesthetised pig. In order to optimize anatomical delineation of the tissue sampling, since the distribution of cell proliferation is highly heterogeneous along the rostro-caudal extent of rodent SVZ (Doetsch et al., 1997; Bauer et al., 2003), we first mapped the proliferative activity within the pig SVZ using Ki-67 immunohistochemistry (Scholzen and Gerdes, 2000; Charrier et al., 2006).

## 2. Methods

### 2.1. Animals, anaesthesia and brain tissue sampling

Fifteen adult, 3–4 month-old Large White Landrace pigs were used in the present study. They were housed with *ad libitum* access to water and standard food pellets, in lodges that comprised one

* Corresponding author at: Physiologie Neurovégétative, UMR 6231 CNRS, Université Aix-Marseille-3, Case 351, Avenue Escadrille Normandie-Niemen, F-13397 Marseille cedex 20, France.
*E-mail address:* emmanuel.moyse@univ-cezanne.fr (E. Moyse).
[1] Both authors contributed equally to the work.

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yard and one hard infrared heated shelter. Anaesthesia and surgery protocols were approved by the local Ethics Committee on the protection of animals used for experimental and other scientific activities with the protocol number 31/2006/IMTSSA/UCPE. Personal protocol numbers for surgery and experimentation with pigs were 2007/29/DEF/DCSSA (to O. Liard) and 2001/102/DEF/DCSSA (to T. Fusaï).

Deep anaesthesia was initiated by intramuscular injection of 30 mg/kg ketamine (Imalgene 1000, Merial, Lyon, France), 0.1 mL/kg acepromazine (Vetranquil, Calmivet, Bayer, Puteaux, France), and 25 μg/kg atropine sulphate (Meram, Melun, France). The animal was then equipped with a peripheral intravenous perfusion of Ringer-lactate-5% glucose serum at 5–7 mL/kg/h, with adhesive electrocardiographic electrodes in CM5-SpO2 (ECG monitor S5, Datex Ohmeda, Madison, WI, USA) and with an artificial oxygenator. Anaesthesia induction was performed by intravenous injections of 2 mg/kg propofol (Diprivan, Bayer) administered slowly over 30 s, and sufentanil (Sufenta, Bayer) at 1.5 μg/kg three times. Local glottis anaesthesia was achieved with 5% lidocaïne spray (Xylocaine, Bayer) before orotracheal intubation with a mono-light cannula (internal diameter 6.5 mm). Ventilation of lung parenchyma was checked with a stethoscope and was mechanically assisted. General anaesthesia was sustained with electrically driven intravenous infusion of propofol 2 mg/kg/h (Diprivan, DCI), sufentanil 1 μg/kg/h (Sufenta, DCI), and rocuronium bromide 0.15 mg/kg/h (Esmeron, DCI). Curarisation was supervised by means of a S5 monitor (Entropie module). Antibioprophylaxy was administered. Throughout surgery, the cardio-ventilatory parameters (heart and ventilation rates, electrocardiogram, blood oxygenation, ventilation pressure and volume, MAC, temperature, hemoglucotest) as well as the sleep threshold were constantly assessed using the S5 monitor.

The dorsal face of the skull of each deeply anesthetized pig was cut by drilling and removed. A 15 mm-thick coronal slice of the brain was separated using a scalpel at the commissural level, manually explanted, and transferred to a 4 °C metal plate on ice next to a Bunsen flame for atmosphere sterilization. The experimental animal was therefore alive until the explantation of the brain tissue slice. Each pig was humanely sacrificed immediately after brain tissue sampling by intravenous injection of Dolethal (R) pentobarbital (DCI) at 1.5 mL/kg.

### 2.2. Histochemistry

For histochemical assays ($n = 2$), the explanted brain slice was fixed by immersion in a 4% paraformaldehyde solution in 0.05 M, pH 7.4, Na $H_2$ $PO_4$/$Na_2$ $HPO_4$ buffer for 24 h at 4 °C, then rinsed for 24 h at 4 °C in 0.1 M, pH 7.4 phosphate-buffered saline (PBS). It was then cryoprotected for 72 h in a 30% sucrose solution, snap-frozen in liquid isopentane at −40 °C, and stored at −80 °C. Coronal 20 μm-thick sections were made in a cryostat (Leica 2800) mounted on 0.05% poly-L-lysine (Sigma) precoated slides, dried 15 h at ambient air, and stored at −20 °C.

### 2.3. Neurosphere assay

For primary cell culture ("neurosphere assay"), a piece of SVZ tissue was microdissected from the pig forebrain slice in low-calcium artificial cerebrospinal fluid (aCSF: 124 mM NaCl, 5 mM KCl, 3.2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 26 mM $NaHCO_3$, 100 mM glucose, pH 7.38, according to Weiss et al., 1996). As previously described (Charrier et al., 2006), the tissue sample was rinsed twice with aCSF, and each SVZ digested first in 40 U cystein-EDTA-βmercaptoethanol-preactivated papain (Sigma, L'Isle D'Abeau, France) for 10 min at 37 °C, then in 250 μL undiluted TrypLE™ Express solution (heat-resistant, microbially produced, purified trypsin-like enzyme, Gibco cat 12604-013, Invitrogen, Cergy-Pontoise, France) for 10 min at 37 °C. After the addition of 750 μL of fresh aCSF and centrifugation for 8 min at 400 × *g* at room temperature, the cell pellet was resuspended in 1 mL culture medium (DMEM [Sigma], 1× B27 [Gibco Invitrogen], 200 U/mL penicillin and 200 μg/mL streptomycin [Gibco Invitrogen]) containing 20 ng/mL Epidermal Growth Factor (EGF, Gibco Invitrogen) and 20 ng/mL basic Fibroblast Growth Factor (bFGF, Gibco Invitrogen). The cells were dissociated gently with a 26G steel needle mounted on a disposable 1 mL syringe, counted on a Malassez slide, and seeded at either 500, 5,000, 10,000, or 30,000 cells (depending upon the specific experimental conditions) in culture medium, either 1 mL per well (24-well plates Corning, Avon, France) or 4 mL par well (6-well plates Falcon, BD Biosciences, Bedford, USA). Cultures were monitored daily to follow the morphological growth of the neurospheres; passage was performed when the majority of spheres was 100–120 μm in diameter. For passage, the primary spheres were collected in sterile tubes, incubated for 45–60 min at 37 °C in 250 μL undiluted TrypLE™ Express solution (Gibco Invitrogen) per 6 mL culture-derived pellet, and dissociated gently with a 26G steel syringe; dispersed cells were centrifuged, counted, and seeded as above. For cell differentiation, spheres were picked with a Pasteur pipette, seeded in wells containing poly-D-lysine (Sigma) precoated sterile coverslips and cultured in the above culture medium in the absence of EGF and bFGF for 7–10 days. All culture media were renewed by the replacement of 500 μL of medium per well every 2–3 days. For immunocytochemistry, coverslips bearing differentiated spheres were rinsed in PBS, fixed in 4% paraformaldehyde-containing PBS for 20 min at 4 °C, and kept in PBS at 4 °C until the assay.

### 2.4. Immunocytochemistry and immunohistochemistry

Immunolabelings were performed on cells and on tissue sections, starting with permeabilization in 0.1 M PBS with 0.5% Triton-X-100. For Ki-67 immunohistochemistry, tissue sections were also treated for 15 min in 3% $H_2O_2$ to quench endogenous peroxidases and for 15 min in 10 mM sodium citrate solution (pH 5.5) at 95 °C to unmask nuclear antigens. After a PBS rinse, all preparations were incubated in blocking buffer (0.1 M PBS, 0.1% Triton, 3% bovine serum albumin, 5% normal goat serum) for 1 h, then overnight at 4 °C with one of the following primary antibodies: mouse anti-Ki-67 (BD Biosciences, Heidelberg, Germany, 550609; 1/600), rabbit anti-GFAP (Dako, Trappes, France Z0334; 1/1000), mouse anti-O4 IgM (Chemicon MAB345; 1/200), mouse anti-ß-III-tubulin (Sigma T8660; 1/150), mouse anti-nestin (BD Biosciences 556309; 1/100), or mouse anti-human nestin (IC1259P, 1/100; Developmental Studies Hybridoma Bank, 1/100; gift of Dr. M. Peschanski, I-STEM, France). Following incubation with primary antibody, the samples were rinsed three times in PBS baths. Immunohistochemical labeling of Ki-67 was revealed by incubations with biotinylated secondary antibodies (Vector Labs; 1/200), streptavidin-peroxidase (ABC Elite kit, Vector), and diaminobenzidine (DAB kit, Vector), then ethanol-dehydration and coverslipping with Depex. All other labelings were revealed by 2 h incubation at room temperature with the appropriate secondary Alexa-fluor-conjugated antibodies (Molecular Probes, Eugene, OR, USA; 1/200), rinsing, and mounting with Vectashield (Vector Laboratories, Burlingame, CA, USA). Fluorescent staining of cell nuclei was performed by 5 min incubation in the dark with DAPI (0.5 μg/mL, Sigma). Multiple fluorescent labelings were performed sequentially. Labeled slides were coverslipped with aqueous mounting medium (Vectashield, Vector, Abcys, France). Immunocytochemical labeling of neurospheres was analyzed with a confocal microscope, with a 20× objective and 1.0 μm-thick confocal planes (LM-510-META, Carl Zeiss, LePecq, France). The relative proportions of the three differentiated cell types were evaluated in 25 triple-labeled spheres by hand-scoring of immunoreactive cells on confocal scans. These raw counts were