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CHAPTER 2

***In Vitro* Analysis of Neuronal Progenitor Cells from Mouse Olfactory Epithelium**

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I INTRODUCTION

In the vertebrate nervous system, neurogenesis – the proliferation of neuronal progenitor cells and differentiation of their progeny into neurons – appears to be a complex, tightly-regulated process involving both extrinsic and intrinsic factors (Calof, 1995). Interpretations of *in vivo* studies of neurogenesis are often rendered problematic by this complexity. Because they allow a more defined level of control and thereby promote a more direct assessment of cause and effect, *in vitro* systems that can recapitulate neurogenesis are an attractive alternative for addressing specific questions concerning this process. These include questions about the complexity of neuronal lineages (i.e. the number of different cell stages involved in generating a differentiated neuron and the relationship among the cells at different stages), as well

as questions concerning the identity of factors that regulate progenitor cell proliferation and neuronal and glial differentiation.

Over several years, our laboratory has contributed to the development of a system for studying mammalian neurogenesis, the olfactory epithelium (OE) of the mouse. The OE is morphologically and functionally similar to the embryonic neuroepithelia that generate the rest of the nervous system, but it has several significant advantages as a system for studying neurogenesis. First, the OE is much simpler in composition than the germinative neuroepithelia of the central nervous system, in that it produces large numbers of only a single type of neuron, the olfactory receptor neuron (ORN). Second, the OE retains both its epithelial morphology and the ability to generate neurons throughout life (Graziadei and Monti Graziadei, 1978). We have taken advantage of these features to establish simple and reliable techniques for generating mouse OE cultures in which neurogenesis occurs efficiently and can be studied quantitatively, as well as to identify several molecular markers for major cell types in the OE (Calof and Chikaraishi, 1989; DeHamer *et al.*, 1994; Gordon *et al.*, 1995; Holcomb *et al.*, 1995; Mumm *et al.*, 1996).

Three different OE culture systems, in which ORN progenitor cells can be identified and studied, are discussed below. The first, OE explant cultures, are initiated as sheets of intact epithelium, purified away from underlying stromal cells during the initial phase of tissue preparation. OE explant cultures contain all cell types present in the OE at the age at which the tissue is dissected (normally day 14–15 of gestation in the mouse), and have been useful for investigating lineage relationships among OE cell types (Calof and Chikaraishi, 1989; Gordon *et al.*, 1995), studying migration of ORNs and their progenitors (Calof and Lander, 1991), and identifying mitogenic effects of growth factors on ORN progenitors (DeHamer *et al.*, 1994). The second culture system consists of a dissociated 'neuronal cell fraction' isolated from OE, and contains solely ORNs and their progenitors. This culture system has been used to study adhesivity of ORNs and their progenitors on different extracellular matrix molecules (Calof and Lander, 1991); to study growth factor effects on proliferation and survival of ORNs and their progenitors (DeHamer *et al.*, 1994; Holcomb *et al.*, 1995); and to investigate the role of apoptosis in the ORN lineage (Holcomb *et al.*, 1995). The third culture system consists of pure OE neuronal progenitor cells, which are isolated by immunological panning. Study of these cultures has provided data concerning putative OE neuronal stem cells, and has also provided evidence for a feedback loop, between ORNs and their progenitors, which regulates neurogenesis in the OE (Mumm *et al.*, 1996). Because detailed methods for preparation of explant and dissociated neuronal cell cultures from embryonic mouse OE have recently been published (Calof *et al.*, 1995), this chapter emphasizes the immunological panning protocol used for isolating, and subsequent studies involving, purified ORN progenitors.

II METHODS AND MATERIALS

All reagents are listed at final concentrations. Unless otherwise noted, all chemicals are from Sigma and all tissue culture reagents are from Gibco-BRL. Dissection and tissue culture solutions are sterilized by autoclaving or by passing through a 0.2 μm

filter, and are stored at 4°C unless otherwise noted. All cultures are incubated in a humidified atmosphere at 37°C, 5% CO₂.

II.1 Explant Culture of Embryonic Olfactory Epithelium

II.1.a Reagents

PBS

Dulbecco's phosphate-buffered saline (Ca²⁺/Mg²⁺ free)

Trypsin–Pancreatin solution

PBS, containing the following, stored at –20°C:

- 30 mg/ml trypsin 1:250 (Difco)
- 10 mg/ml porcine pancreatin (Sigma)
- 100 U/ml penicillin–streptomycin

Post-trypsin rinse solution

PBS, containing:

- 0.25 mg/ml trypsin inhibitor, Type I-S from soybean (Sigma, T-9003)
- 3 mg/ml glucose
- 0.5 mg/ml crystalline bovine serum albumin (BSA; ICN Biomedicals, 103700)
- 50 U/ml penicillin–streptomycin
- 5 µg/ml phenol red

Trituration medium

PBS, containing:

- 3 mg/ml glucose
- 50 U/ml penicillin–streptomycin
- 5 µg/ml phenol red
- 0.5 mg/ml crystalline BSA

10 × EBSS

10 × Earle's Balanced Salt Solution (Ca²⁺/Mg²⁺-free)
Double-distilled H₂O containing:

- 4 g/l KCl
- 68 g/l NaCl

- 1.4 g/l $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$
- 10 g/l D-glucose
- 100 mg/l phenol red

Ca²⁺-free DMEM

Ca²⁺-free Dulbecco's Modified Eagle's Medium

Dilute 10 × EBSS to 1 × in double distilled H₂O containing:

- 3.5 mg/ml glucose
- 2.2 mg/ml NaHCO_3
- 0.2 mg/ml $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
- 10 μg/l $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$
- 1 × MEM vitamins (Gibco-BRL, 11120-052)
- 1 × MEM essential amino acids without L-glutamine (Gibco-BRL, 11130-051)
- 1 × MEM non-essential amino acids (Gibco-BRL, 11140-050)
- 5 μg/ml phenol red

LCM

A defined serum-free low-Ca²⁺ medium (final Ca²⁺ concentration 0.1 mM) is used for all OE explant and neuronal cultures described below. LCM is a mixture of two parts Ca²⁺-free DMEM to one part Ham's F12 medium with L-glutamine, containing:

- 100 μM putrescine
- 10 μg/ml insulin
- 10 μg/ml holo-transferrin (Sigma, T-1283)
- 20 nM progesterone
- 30 nM selenium
- 50 U/ml penicillin-streptomycin
- 2 mM L-glutamine
- 5 mg/ml crystalline BSA [or 1 mg/ml clinical reagent grade BSA (CRG-BSA, ICN, 105033)] where noted

Neutralize with 1 M NaOH, adding dropwise until medium is restored to neutral pH.

10% formalin

Formalin is a stabilized 37% formaldehyde solution. For fixation of explant cultures formalin is diluted 1:10 (v/v) in PBS and sucrose is added to a final concentration of 5%. Cultures are fixed by gently underlaying tissue culture medium with the denser, sucrose-containing fixative in order to avoid tissue loss. Typically cultures are fixed for 10 min at room temperature (the fixative is warmed to 37°C before use) and then rinsed with PBS prior to further processing.

II.1.b Tissue preparation

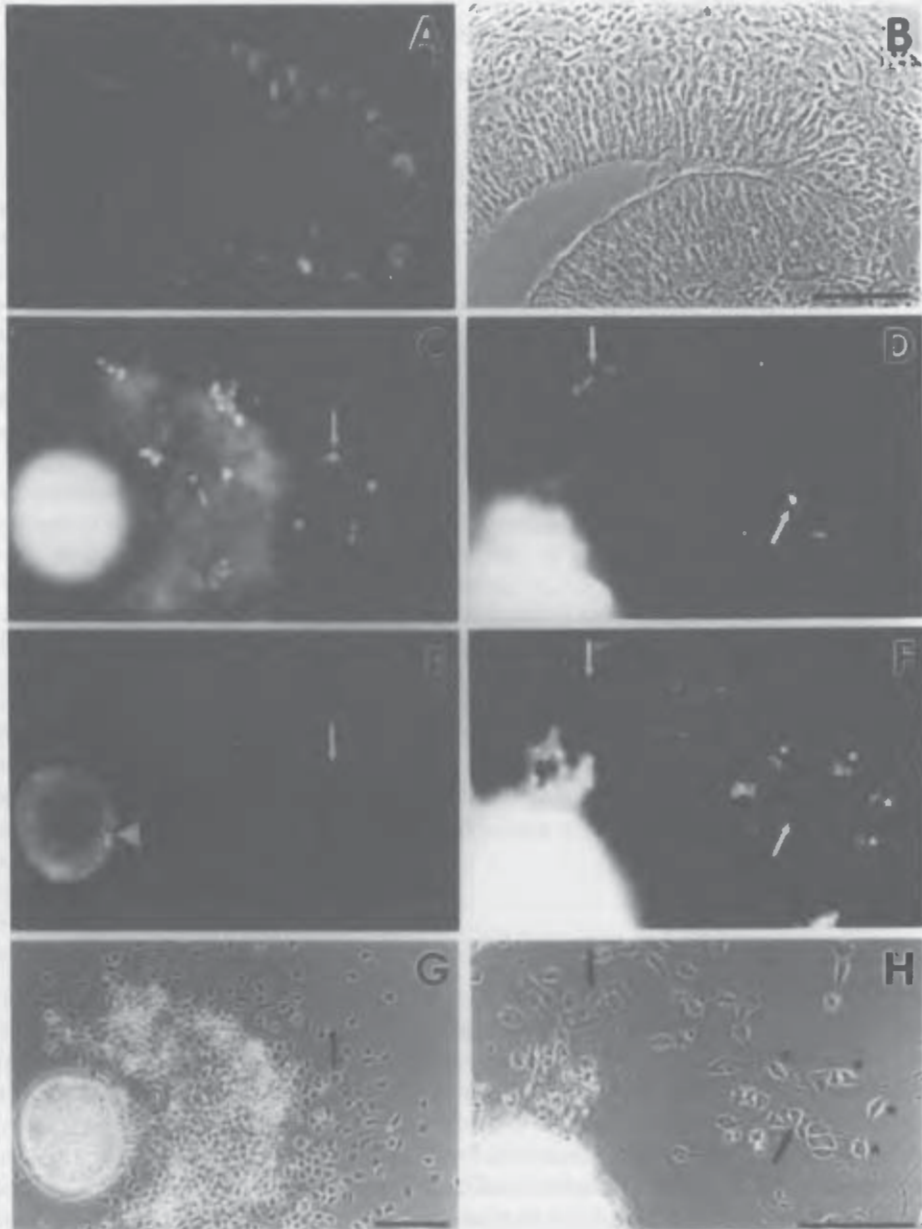
For this and all procedures described below, OE is dissected and purified from mouse embryos taken at day 14.5–17.5 of gestation. Mice are naturally mated, with day of vaginal plug detection denoting embryonic day 0.5 (E 0.5).

1. Embryos are dissected in cold PBS, and developing nasal turbinates are isolated and incubated on ice in trypsin–pancreatin solution (40–45 min, see Calof *et al.*, 1995 for detailed methods).
2. The turbinates are rinsed in post-trypsin rinse solution and placed into small dishes of trituration medium. OE tissue is separated from underlying stroma by repeated trituration using a hand-held, flame-polished Pasteur pipette controlled by a mouth pipettor.
3. Using a transilluminating dissecting microscope equipped with dark-field optics, translucent 'sheets' of OE tissue are purified from the more opaque pieces of stroma and clean pieces of pure OE are removed to new dishes containing LCM.
4. Purified OE is transferred to new LCM dishes and inspected for stromal contamination a minimum of two times before placing into culture.
5. For explant cultures, purified OE sheets are chopped into small pieces using a tungsten wire 'knife' (etched in 10% KOH). The finely chopped OE pieces are then distributed onto acid-washed (1% HCl), UV-sterilized, glass coverslips placed into individual wells of 24-well tissue culture trays containing 1–2 ml of LCM per well.

Some coating of the coverslips is required to promote attachment of OE explants and migration of ORNs and neuronal progenitors onto the tissue culture substratum. One common coating that maximizes cell migration consists of sequential treatment of coverslips with poly-D-lysine (1 mg/ml in water) followed by merosin (10–20 µg/ml in CMF-HBSS) (DeHamer *et al.*, 1994). OE tissue isolated from six to eight turbinates per coverslip is required for optimum density, and explants should adhere and cell migration initiate within 6 h of plating.

II.1.c Stage-specific markers for ORNs

Explant cultures of embryonic mouse OE were developed as a system for characterizing the cellular and molecular regulation of neurogenesis (Calof and Chikaraishi, 1989). For such a system to be most useful, it has been necessary to find antibody markers that distinguish among cell types of the OE. Several antibodies have been found to be particularly useful: a commercially available antiserum to keratins (Dakopatts Z622) stains the horizontal basal cells of the OE, while two different monoclonal antibodies to the neural cell adhesion molecule, NCAM [AG1 mouse anti-NCAM and H28 rat anti-NCAM (Calof and Chikaraishi, 1989; DeHamer *et al.*, 1994)], react specifically with post-mitotic ORNs. A monoclonal antibody to the transcription factor MASH1 has also been instrumental in showing that expression of MASH1 identifies a distinct stage of ORN progenitor, and in placing MASH1-expressing progenitors within the ORN lineage (Gordon *et al.*, 1995). Optimal fixa-



tion conditions and antibody detection methods differ for each of these antibodies, and details of their use have been published (Calof *et al.*, 1995; Gordon *et al.*, 1995). An example of this type of immunocytochemical analysis of OE explant cultures is illustrated in Figure 1.

II.1.d S-phase labelling

For identification of proliferating cells, explant cultures are treated with reagents that label cells in the S-phase of the cell cycle. For pulse-fix and pulse-chase experiments, in which cell cycle parameters need to be measured with precision, this is normally a short (2-h) pulse of a high final concentration of labeling reagent [either ^3H -thymidine (^3H -TdR, 5 $\mu\text{Ci/ml}$, 80 Ci/mmol, New England Nuclear) or bromodeoxyuridine (BrDU, 1:1000 dilution of cell proliferation labeling reagent #RPN201, Amersham)] (Calof and Chikaraishi, 1989; DeHamer *et al.*, 1994). In experiments in which it is desirable to label the maximum number of proliferating cells within a population in which progenitor cells are at varied stages of the cell cycle, a longer application of labelling reagent is necessary. However, in these situations it is necessary to reduce the concentration of labelling reagent to avoid toxicity. For example, for experiments in which neuronal progenitors are labeled for 12 or more hours, ^3H -TdR is reduced to 0.1 $\mu\text{Ci/ml}$ final concentration and BrDU to a final 1:10 000 dilution of the commercially obtained stock (DeHamer *et al.*, 1994).

Figure 1 MASH1 expression in cells of the olfactory epithelium (OE) *in vivo* and *in vitro* (A,B) Embryonic day 15.5 mouse OE was sectioned (cryostat sections, 12 μm) and processed for MASH1 immunoreactivity. (A) Viewed in rhodamine optics, a section through a developing nasal turbinate shows scattered MASH1⁺ cells throughout the OE, particularly near the basal lamina. (B) Viewed under phase-contrast optics, the same field shows the entire epithelium and some of its underlying stroma. Scale bar, 50 μm . (C–H) Embryonic OE explants were fixed and processed for double-label immunofluorescence for MASH1 and keratin (C, E, G) or for MASH1 and N-CAM (D, F, H) following 10-h culture. (C) MASH1 immunoreactivity (arrow indicates MASH1-expressing cell) shown under rhodamine optics. (D) Immunoreactivity (arrows indicate MASH1-expressing cells) in a different field shown under rhodamine optics. (E) Keratin immunoreactivity in the same field as C (large arrowhead indicates keratin-expressing cells in the body of the explant, arrow indicates position of MASH1-expressing cell shown in C) viewed in fluorescein optics. (F) NCAM immunoreactivity in the same field as D (asterisks indicate NCAM-expressing cells, arrows indicate positions of MASH1-expressing cells shown in D) viewed in fluorescein optics. (G) The same field as in (C) and (E), viewed under phase-contrast optics [arrow indicates the MASH1-positive/keratin-negative cell shown in (C) and (E)]. (H) The same field as in (D) and (F), viewed under phase-contrast optics [arrows indicate the MASH1-positive/NCAM-negative cells shown in (D) and (F), asterisks indicate the NCAM-positive cells shown in (F)]. Scale bars, 50 μm . [Adapted with permission from Gordon *et al.* (1995), copyright 1995 Academic Press.]

Autoradiography

³H-TdR incorporation by cells is detected using emulsion autoradiography. This is most conveniently performed by having first grown the OE explant cultures on glass coverslips, which can be mounted on glass slides to facilitate autoradiographic dipping and developing:

1. Fix cultures with 10% formalin fixative for 10–15 min at room temperature, dehydrate and dip in NTB2 emulsion (Kodak) diluted 1:1 in water.
2. Expose at –80°C (48 h for 5 µCi/ml pulses; 7–8 days for 0.1 µCi/ml pulses).
3. Develop with D-19 developer (Kodak) according to manufacturer's instructions.

BrDU incorporation and immunocytochemistry

There are many different commercially obtainable anti-BrDU antibodies, and the investigator should choose the one most suitable to the culture system and to other labelling requirements of the particular experiment. One suggestion can be found in the paper by DeHamer *et al.* (1994). Because no dipping is required for detection of BrDU incorporation, cultures can be grown in multiwell trays and other types of plastic tissue culture dishes, as well as on glass coverslips, as long as secondary antibodies labelled with enzymes (HRP, alkaline phosphatase) are used to detect anti-BrDU primary antibodies (glass coverslips are still the most convenient growth surface for immunofluorescence experiments).

Detailed discussions of the use of ³H-TdR and BrDU labelling to determine progenitor cell responsiveness to different growth factors and to estimate cell cycle parameters of ORN progenitors have recently been published, and the reader is referred to this manuscript for more information (DeHamer *et al.*, 1994).

II.1.e Combined labelling methods

Both BrDU and ³H-TdR incorporation can be used in combination with cell type-specific antibody markers, allowing both for quantitative analysis of differentiation of progenitor cells (Calof and Chikaraishi, 1989), and for identification of progenitor cell-specific markers (Gordon *et al.*, 1995) in OE explant cultures¹. This method has been used to identify the Immediate Neuronal Precursors (INPs) of ORNs, and to show that INPs quantitatively give rise to postmitotic, NCAM-expressing ORNs within ~15 h of their terminal S-phase (Calof and Chikaraishi, 1989). Triple-labelling experiments, in which cells are labelled by both BrDU and ³H-TdR S-phase markers, as well as a cell type-specific antibody marker, are also possible as long as:

¹ In OE explant cultures, progenitor cell proliferation and differentiation are quantified by examining only the migratory neuronal cells, i.e. ORNs and neuronal progenitors that leave the body of the explant and disperse onto the culture substratum. We limit our analysis to migratory neuronal cells because (a) all or most ORNs and ORN progenitors appear to have the ability to migrate; and (b) the proportion of ORNs to progenitors appears to be the same in the migratory cell fraction as in OE cultures as a whole (Calof and Chikaraishi, 1989; Calof and Lander, 1991). (c) Finally, the dispersal of migratory cells enables their numbers to be counted and their phenotypes assessed accurately.

(i) the antibody to BrDU and the antibody to the cell-type marker are compatible for double-labelling experiments; and (ii) immunocytochemistry is performed prior to autoradiographic processing. In addition, since most BrDU detection procedures require use of either acid or base to strip histones and denature DNA prior to incubation with primary antibody, the investigator must determine whether the antigen to be detected with the cell type-specific marker can withstand this harsh treatment. Triple-label experiments, detecting both $^3\text{H-TdR}$ and BrDU incorporation as well as NCAM expression, have been used to demonstrate that fibroblast growth factors promote multiple rounds of division by INPs prior to terminal differentiation of these cells' progeny into NCAM-expressing ORNs (DeHamer *et al.*, 1994).

II.2 Dissociated Neuronal Cell Culture of Olfactory Epithelium

II.2.a Media and reagents

Dissociation and purification of the neuronal cell fraction from OE explant suspension cultures will require the following additional reagents to those described above.

HBSS

$\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hank's balanced salt solution.

Trypsin solution

5 mg/ml trypsin (Sigma, T-8253) in Leibovitz's L-15 medium, store at -20°C .

DNase

1 mg/ml deoxyribonuclease I (Sigma, type IV, D-5025) in DMEM, store at -20°C .

Trypsin inhibitor

5 mg/ml trypsin inhibitor from soybean (Sigma, type I-S) in Leibovitz's L-15 medium, store at -20°C .

4% BSA

40 mg/ml crystalline BSA in HBSS, store at -20°C .

II.2.b Tissue preparation

1. Pieces of purified OE (see Section II.1.b), isolated from E14.5–E17.5 mouse embryos (age used depends on the experiment), are incubated in suspension culture in 60-mm Petri dishes in LCM containing 5 mg/ml crystalline BSA for 6–12 h. The pieces of OE do not attach to the Petri plastic. During the incubation period, ORNs and ORN progenitors sort out from (but still remain loosely attached to) the basal epithelial cells of the OE, which remain in tightly associated sheets.
2. Following the incubation period, the entire contents of the suspension culture are removed from the Petri dish and pelleted by centrifugation at $100 \times g$ for 5 min.
3. The supernatant is discarded and the tissue is gently resuspended in 4 ml HBSS.
4. 0.4 ml of trypsin solution is added and the tube is incubated at 37°C for 4 min.
5. 1 ml of DNase solution is then added and the tube is returned to 37°C for 6 min.
6. 1 ml of the trypsin inhibitor solution is added to stop proteolytic digestion, and the neuronal cells are mechanically dissociated by triturating 10 times using a flame-polished Pasteur pipette.
7. The large fragments of tightly associated basal epithelial cells are allowed to settle out of solution (3 min), and the supernatant (~5 ml) is carefully removed and filtered through sterilized nylon mesh (10–20 μm pore size; Small Parts Inc., Miami, FL) into a separate tube.
8. 5 ml of HBSS containing 1 mg/ml trypsin inhibitor, plus 1 ml of DNase solution, are added to the remaining fragments and the trituration, settling, and filtration procedures are repeated.
9. The pooled supernatants are underlaid with a solution of 4% crystalline BSA in HBSS and centrifuged at $100 \times g$ for 10 min.
10. The pelleted cells ('neuronal cell fraction') are then resuspended in LCM containing 5 mg/ml crystalline BSA and plated according to the requirements of the particular experiment.

An advantage of dissociated cell cultures over explant cultures is that the number of cells initially plated in the experiment is known, making it possible to assess effects of different culture treatments by counting the total number of cells showing a particular effect, as well as by assessing the percentage of cells showing a particular effect in randomly chosen fields in the culture.

II.2.c Stage-specific markers for ORNs

The purity of the neuronal cell fraction isolated by this method is assessed by immunocytochemistry of the various cell fractions using cell type-specific antibodies. In studies in our laboratory, the neuronal cell fraction has been found to contain solely NCAM⁺ ORNs and NCAM⁻ progenitor cells, with < 1% contamination by keratin-expressing horizontal basal cells (Calof and Lander, 1991). Depending on the incubation period of the suspension culture, the neuronal cell fraction contains anywhere from 60–75% differentiated, NCAM⁺ neurons, with the remainder consisting

of NCAM⁻ progenitor cells (Calof and Lander, 1991; Holcomb *et al.*, 1995; Mumm *et al.*, 1996).

Because dissociated neuronal cell cultures do not contain horizontal basal cells and sustentacular cells of the OE, these cultures are particularly useful for assessing direct effects of growth factors and pharmacological agents on ORNs and their progenitors. Use of these cultures eliminates the likelihood that observed effects are mediated indirectly through the non-neuronal cell types present in explant cultures. For cultures in which growth factor effects on OE neuronal cells are tested, cells are routinely plated at varying densities in 96-well tissue culture trays that have been coated with poly-D-lysine (1 mg/ml in water) and then sterilized by UV irradiation. Cultures are grown for 12–72 h, and then fixed using the 10% formalin fixative described in Section II.1.a.

Analysis of OE neuronal cells cultured in 96-well trays is limited to morphological assays and the use of single molecular markers that can be detected with enzymatic/histochemical assays to assess differentiation state, BrDU incorporation, or apoptosis (TUNEL staining; cf. Holcomb *et al.*, 1995). Despite this limitation, this culture system has proved extremely useful for analysis of ORNs and their progenitors. For example, dissociated neuronal cell cultures have been used to confirm that mitogenic effects of FGFs are exerted directly on ORN progenitors, rather than being mediated indirectly through other cell types in OE explant cultures (even though FGF effects on OE progenitors are readily detectable in explant cultures; DeHamer *et al.*, 1994). In this same study, the dissociated neuronal cell preparation was used in reverse transcriptase–polymerase chain reaction analyses to identify the FGF tyrosine kinase receptors expressed by OE neuronal cells (DeHamer *et al.*, 1994).

II.2.d Regulation of survival

For identifying neurotrophic factors and pharmacological agents that promote survival of ORNs and ORN progenitors, dissociated neuronal cell cultures have been essential. In such experiments, the most sensitive assay for determining survival-promoting effects of different agents has been to grow the cells in 96-well tissue culture trays, as described above, and then count the total number of living, neurite-bearing ORNs present in cultures that have received different treatments. An example of this type of culture experiment, in which OE neuronal cells were grown in the presence and absence of aurointricarboxylic acid (an agent that blocks apoptosis), is shown in Figure 2. If dissociated neuronal cells are plated onto glass coverslips rather than in tissue culture trays, ³H-TdR labelling can be combined with both TUNEL histochemistry (end-labelling of fragmented DNA with biotinylated dUTP to detect apoptotic cells) and antibody staining to determine which cell types in the ORN lineage are undergoing apoptosis and whether particular treatments promote their survival. This type of analysis has been used to demonstrate that ORN progenitors, as well as differentiated ORNs, undergo apoptosis, and that aurointricarboxylic acid can ameliorate this and promote survival of cultured ORN progenitor cells (Holcomb *et al.*, 1995).

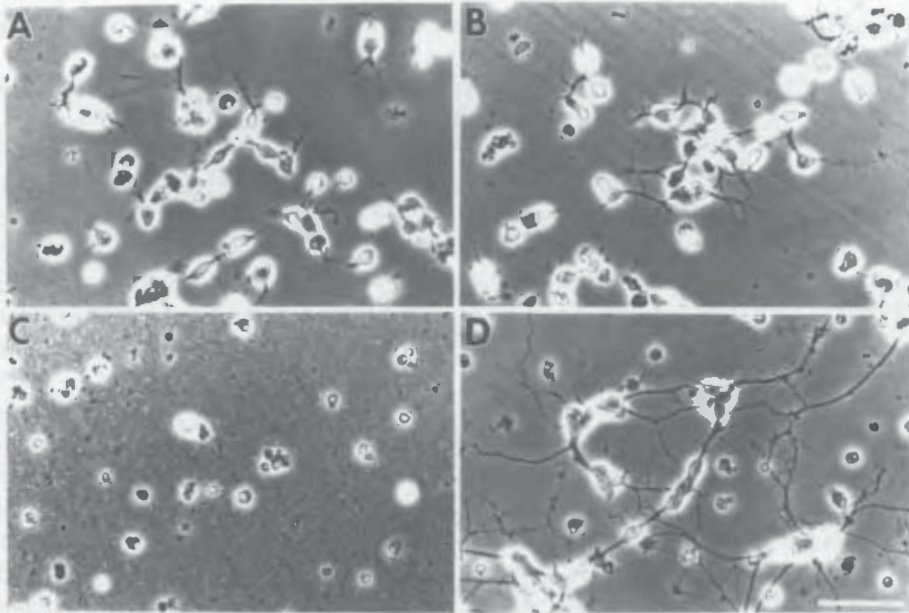


Figure 2. Inhibition of programmed cell death (apoptosis) in olfactory neuronal cells by aurintricarboxylic acid (ATA) *in vitro*. Dissociated OE neuronal cells plated at $\sim 4 \times 10^4$ cells/well in 96-well tissue culture plates in LCM plus 5 mg/ml crystalline BSA, with or without ATA (100 μ M), viewed under phase-contrast optics. (A) 24 h *in vitro*, control. (B) 24 h, + ATA. (C) 72 h *in vitro*, control. (D) 72 h, + ATA. Scale bar, 50 μ m. ATA promotes survival of a significant fraction of cultured ORNs. [Adapted with permission from Holcomb *et al.* (1995), Copyright © 1995 Academic Press.]

II.3 Cultures of Purified Neuronal Progenitors

II.3.a Media and reagents

The immunological panning procedure and purified neuronal progenitor cultures will require the following reagents in addition to those described above.

H28 rat anti-NCAM antibody

Use either supernatant harvested from H28 hybridoma cells (obtained from Dr. Christo Goridis, INSERM-CNRS, Marseilles, France) as described below or the purified antibody (Immunotech Inc., 1-800-656-3497; Cat. No. 0270) at 1 μ g/ml in Tris-buffered saline (pH 7.5).

Hybridoma medium

RPMI 1640 medium, containing:

- 10% fetal bovine serum (Hyclone, A-1111-L)
- 5 mM HEPES, pH 7.2
- 2 mM L-glutamine
- 1 mM sodium pyruvate
- 100 U/ml penicillin–streptomycin

Serum-free hybridoma medium

Hyclone, HyQ-CCM™1

Goat anti-rat IgG

50 µg/ml (Cappel #55722) in 50 mM Tris buffer, pH 9.5

Dispase (stock solution)

1 mg/ml dispase (Boehringer-Mannheim Grade I, >6 U/mg) in Leibovitz's L-15 medium, store at –20°C in 250 µl aliquots

Panning medium

Leibovitz's L15 medium, containing:

- 1 mg/ml crystalline BSA
- 3 mg/ml glucose
- 10 µg/ml insulin
- 10 µg/ml holo-transferrin
- 20 nM progesterone
- 100 µM putrescine
- 30 nM selenium
- 40 U/ml penicillin–streptomycin

Stroma medium

DMEM (high glucose formulation), containing:

- 10% defined, supplemented newborn calf serum (Hyclone)
- 2 mM L-glutamine
- 100 U/ml penicillin–streptomycin

X-gal fixative

For histochemistry to detect *lacZ*-expressing cells (see below). PBS (pH 7.5), containing:

- 0.5% glutaraldehyde
- 5% sucrose
- 2 mM MgCl₂

X-gal wash

PBS (pH 7.5), containing 2 mM MgCl₂

X-gal detergent

X-gal wash, containing

- 0.1% Triton
- 0.01% deoxycholic acid

X-gal mix

X-gal detergent, containing

- 5 mM K₃Fe(CN)₆
- 5 mM K₄Fe(CN)₆
- 320 µg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)

II.3.b Tissue preparation

An immunological panning method is used to purify NCAM-negative OE neuronal progenitors away from NCAM-expressing ORNs present in the dissociated neuronal cell fraction described in Section II.2. For this procedure, antibodies that recognize an extracellular epitope on the NCAM molecule are bound to a Petri dish, and the dissociated neuronal cell fraction is incubated on the dish, allowing NCAM-expressing ORNs to be captured by the anti-NCAM antibodies. Unattached, NCAM-negative progenitor cells are then removed and cultured as described below.

Immunological Panning to Purify Progenitors

1. Serum-free supernatant (200 ml) from H28 rat anti-NCAM hybridoma cells is precipitated in 50% saturating ammonium sulphate as described (Mumm *et al.*, 1996).

2. Precipitated antibody is pelleted by centrifugation for (30–60 min at $3000 \times g$) and the undialysed precipitate is resuspended in 30 ml of HBSS and stored in 5 ml aliquots at -80°C .
3. The panning plate is prepared by coating a 100-mm Petri dish (Fisher #08-757-13) with 5 ml of $50 \mu\text{g/ml}$ goat anti-rat IgG (Cappel #55722) in 50 mM Tris buffer (pH 9.5) overnight at 4°C .
4. After rinsing three times with HBSS, 5 ml of concentrated H28 precipitate is added to the plate, which is then incubated at 4°C from 8–12 h.
5. One hour before use, the panning plate is rinsed extensively with HBSS and blocked in 10 mg/ml CRG-BSA in HBSS. The dissociated neuronal cell fraction is isolated from E14.5–15.5 OE suspension cultures as described in Section II.2.b, except that dispase ($50 \mu\text{g/ml}$ for 10 min at 37°C) is used instead of trypsin for cell dissociation.
6. The OE neuronal cell fraction is collected by pelleting through a cushion of 4% crystalline BSA in HBSS (10 min at $100 \times g$), resuspended in 5 ml of panning medium, and incubated on the panning plate for 30 min at room temperature in the dark with intermittent agitation.
7. The purified progenitor cells, which remain in suspension, are collected, centrifuged for 5 minutes at $100 \times g$, resuspended in LCM, plated at desired densities and incubated.

For progenitor cell–stroma co-cultures (see below), the stromal tissue subjacent to the OE, which is normally discarded during the course of OE purification (see Section II.1), is instead retained. Stromal fibroblasts are dissociated to a single cell suspension by mechanical trituration through a flame-polished Pasteur pipette. The stromal cell suspension is then passed through a $20 \mu\text{m}$ nylon mesh filter to remove aggregates, pelleted at $100 \times g$ for 5 min, and plated in stroma medium on 100-mm tissue culture plates. To generate mitotically inactivated feeder layers, stromal cells are grown to about 80% confluence, harvested, and irradiated in suspension (3300 Rad). The cells are then collected by centrifugation, resuspended in stroma medium, and plated in 96-well tissue culture trays (Costar #3596) at a density of $3\text{--}4 \times 10^4$ cells per well the day prior to the experiment. Approximately 1 h before plating purified progenitors over them, feeder layers are rinsed with CMF-HBSS and switched into LCM. Purified progenitor cells, isolated from ROSA26 transgenic mice (see below), are typically added at a density of 1000 cells per well in 96-well plates and cultures are incubated as described (Mumm *et al.*, 1996). Figure 3 is a flow chart illustrating the panning and co-culture procedures.

II.3.c Experimental analysis

To verify the purity of progenitor cell preparations, an independent NCAM antibody (AG1D5) can be used. Cells from 'before' and 'after' panning fractions are plated onto poly-D-lysine coated coverslips, incubated for 30 min at 4°C , fixed in Omnifix II (An-Con Genetics) for 15 min and processed as described for AG1D5

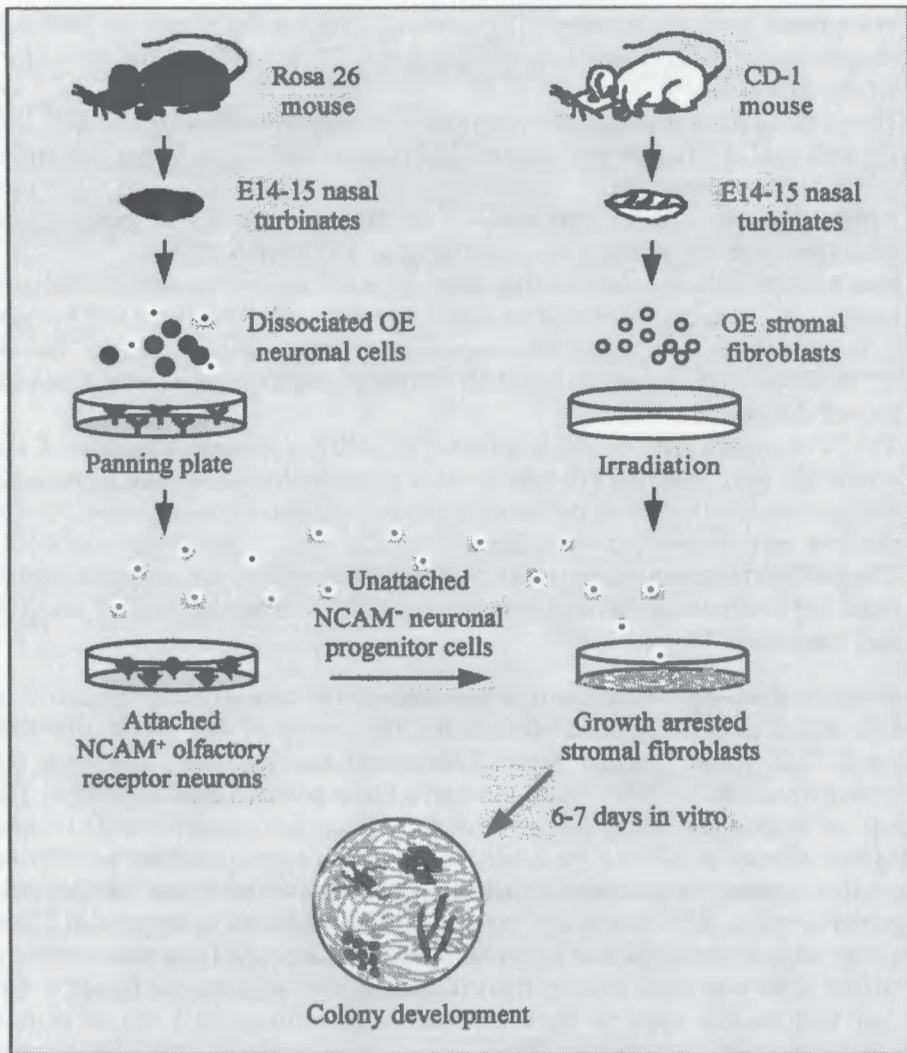


Figure 3 Methods to purify neuronal progenitor cells from the OE using a negative selection procedure. OE progenitors are purified from ROSA26 mice, which express a *lacZ* transgene in all cells, allowing purified cells to be detected using Xgal histochemistry or antibodies to β -galactosidase. Since post-mitotic ORNs express NCAM, whereas ORN progenitors do not, immunological panning is used selectively to remove NCAM-expressing ORNs from a dissociated neuronal cell fraction that consists entirely of NCAM⁺ ORNs and NCAM⁻ progenitors. The progenitor cell fraction obtained from this procedure is typically 95–99% pure (assessed by anti-NCAM staining). This progenitor cell fraction is then co-cultured with growth-arrested stromal cells in serum-free medium. This figure is a flow chart illustrating the procedure

(DeHamer *et al.*, 1994). Typically, the post-pan suspension consists of >96% NCAM-negative cells (Mumm *et al.*, 1996).

Purified progenitor cell suspensions can be plated onto polylysine/merosin-coated substrata in LCM and their proliferation, differentiation, and survival assessed using the same approaches described above for other types of cultures. Such studies have been used to verify that the majority of these purified progenitor cells behave in a manner consistent with their identification as Immediate Neuronal Precursors (INPs) of ORNs: in these defined culture conditions, over 95% of purified progenitors differentiate into NCAM-expressing ORNs within the first 2 days of culture; by 7 days in culture, the newly generated ORNs and remaining undifferentiated progenitors have died, probably due to lack of environmental trophic support (DeHamer *et al.*, 1994; Holcomb *et al.*, 1995; Mumm *et al.*, 1996).

II.3.d Identification of progenitor cells in feeder-layer cultures

A search for culture conditions which promote the survival and continued proliferation of purified ORN progenitor cells led to the development of progenitor cell-stroma co-cultures, which have since been used routinely. In these cultures, purified progenitors are grown on a growth-arrested confluent 'feeder layer' of OE stromal fibroblasts. This necessitates a means of visualizing the OE progenitor cell population in the co-cultures. Our solution to this problem has been to isolate OE progenitor cells from embryos of the ROSA26 transgenic mouse strain (TgR[ROSA26]26Sor, Jackson Laboratory, Bar Harbor, ME), while the stromal fibroblasts on which the progenitor cells are cultured come from outbred Swiss mice (CD-1 strain, Charles River Breeding Laboratories). ROSA26 mice express a bacterial β -galactosidase transgene in all cells, allowing ROSA26-derived cells to be identified using X-gal histochemistry or antibodies to bacterial β -galactosidase.

To visualize β -galactosidase-expressing cells using X-gal histochemistry, co-cultures are fixed in X-gal fixative for 15 min, rinsed twice with X-gal wash, incubated for 1 h at room temperature in X-gal detergent, and then stained with X-gal mix for 1–2 days at 37°C. Four different morphologically identifiable types of colonies arise from ROSA26-derived progenitor cells in these cultures: spindle-shaped cell colonies; medium-round cell colonies; polygonal fibroblastic cell colonies; and colonies containing small, round cells, many of which bear neuritic processes. Only this last type of colony can be shown, using anti-NCAM immunocytochemistry, to contain NCAM-expressing ORNs, and so these have been termed 'neuronal' colonies (Mumm *et al.*, 1996). Pulse-chase ^3H -TdR incorporation experiments can also be used to study proliferation and differentiation in neuronal colonies, provided that progenitor cell-stroma co-cultures are grown on glass coverslips (Mumm *et al.*, 1996, see Plate 3). Such studies, as well as statistical analyses of neuronal colony development, have led to the hypothesis that 'neuronal colony-forming cells' may be the neuronal stem cells of the OE (Mumm *et al.*, 1996). Our ongoing studies of these cells are focused on using the progenitor cell-stroma co-culture system to identify cell interactions and molecular factors that regulate neurogenesis by neuronal colony-forming cells (see below and Mumm *et al.*, 1996).

III APPLICATIONS

The tissue culture systems described above were developed for studying the molecular regulation of neurogenesis in mouse OE. Initially, explant cultures of OE purified from E14.5–15.5 mouse embryos were used to identify molecular markers for major cell types in the OE: antibodies to the neural cell adhesion molecule NCAM were shown to mark post-mitotic, differentiated olfactory receptor neurons (ORNs), and a commercial antiserum to keratins was shown to mark horizontal basal cells (Calof and Chikaraishi, 1989). In such studies, it was observed that cultured OE explants rapidly adhere to appropriate culture substrata, and within 6 h a population of cells rapidly sorts out from the main body of the explant (in which the keratin⁺ horizontal basal cells remain) and begins to migrate out onto the substratum (Calof and Lander, 1991). Virtually all of these migratory cells were found to be either NCAM⁺ ORNs or proliferating cells that incorporated ³H-TdR (Calof and Chikaraishi, 1989; Calof and Lander, 1991). Over the course of 2 days in culture, the ORNs and proliferating cells continue to migrate, during which time nearly every proliferating cell divides once to give rise to two daughter cells that both differentiate into NCAM-expressing ORNs. This event was followed experimentally by first, pulse-labelling proliferating cells in explant cultures with ³H-TdR, and then following the acquisition, by ³H-TdR-labelled cells, of the ORN-specific NCAM marker (Calof and Chikaraishi, 1989). These and other experiments demonstrated that neurogenesis occurs efficiently in OE cultures, and identified the direct progenitor of ORNs, which we have called the INP (Calof and Chikaraishi, 1989).

Studies by others had suggested that INPs *in vivo* could undergo two to three rounds of division before their progeny differentiated into ORNs, but initially only one round of INP division was observed in explant cultures. However, by screening for molecules that prolong INP proliferation in OE cultures, members of the Fibroblast Growth Factor family (FGFs) were identified as stimulators of INP cell divisions (DeHamer *et al.*, 1994). That multiple divisions of INPs occur in FGF2 was demonstrated conclusively using sequential labelling with BrDU and ³H-TdR specifically to mark ORNs that had been generated as a result of two successive rounds of cell division in culture. In these experiments, the incidence of such double-labelled ORNs was found to be four- to five-fold greater in FGF2-treated cultures than in controls (DeHamer *et al.*, 1994). Importantly, FGFs did not alter the fate of INPs; virtually all INPs still gave rise to ORNs, even with FGFs present. Thus, as committed neuronal progenitor cells, capable of undergoing a limited number of divisions in response to exogenous factors, INPs fit the description of 'transit amplifying cells', a term used to describe intermediate-stage progenitors in other lineages (e.g. the haematopoietic lineage) (DeHamer *et al.*, 1994).

Further studies of explant cultures revealed additional complexity in the ORN lineage. These studies were prompted by the finding that mice with disruption of both alleles of the gene encoding Mammalian Achaete Scute Homolog 1 (MASH1) show a near-total absence of ORNs, as well as certain autonomic and enteric neurons (Guillemot *et al.*, 1993). Using OE explant cultures, it was found that MASH1 is expressed by neuronal progenitor cells at a distinct stage in the ORN lineage, a stage upstream of INPs (Gordon *et al.*, 1995). Evidence supporting this conclusion

came partly from observing the rapid disappearance of MASH1⁺ cells from OE explant cultures; this disappearance appears to reflect the division MASH1⁺ cells to generate MASH1⁻ INPs (Gordon *et al.*, 1995). Additional observations of MASH1-expressing cells both in OE explant cultures and *in vivo* led to the conclusion that MASH1⁺ cells are not stem cells, but like INPs, act as neuronal transit amplifying cells (Gordon *et al.*, 1995). These findings were significant for two reasons: they indicated that neuronal lineages are more complex than had previously been appreciated; and, taken together with the findings from *Mash1* 'knockout' mice, they suggested that an important consequence of lineage complexity was the opportunity for cell number to be regulated at each developmental stage (Calof, 1995).

Although OE explant cultures have been very helpful for identifying stages in the ORN lineage, they have some drawbacks for mechanistic studies. For example, because explant cultures contain cell types other than migratory ORNs and neuronal progenitors (primarily keratin⁺ horizontal basal cells, which do not migrate), it is always possible that the effects of added factors (e.g. growth factors) on progenitors might be exerted indirectly, e.g. through horizontal basal cells. For this reason, dissociated OE neuronal cell cultures were developed. The 'neuronal cell fraction' isolated from suspension cultures of purified embryonic OE, described above, has been shown to consist entirely of NCAM⁺ ORNs and NCAM⁻ ORN progenitors, in a ratio of approximately 3:1, without contaminating keratin⁺ horizontal basal cells, and can be used either for biochemical analysis or growth in culture. Cultures of dissociated OE neuronal cells have been used to demonstrate that FGF2's effects on INP cell divisions are direct (DeHamer *et al.*, 1994); to analyse FGF receptor expression by ORNs and progenitors (DeHamer *et al.*, 1994); and to study apoptotic death of cells in the ORN lineage and the regulation of ORN survival by neurotrophins (Holcomb *et al.*, 1995).

In addition, the dissociated neuronal cell fraction is used as a starting point for preparing cultures of purified OE neuronal progenitor cells. The OE has long been thought to harbour a neuronal stem cell because generation of ORNs takes place continually throughout life, and studies of purified progenitor cell cultures were initiated originally with the goal of obtaining more direct evidence for the existence of OE stem cells (Mumm *et al.*, 1996). These studies showed that purified progenitors rapidly undergo terminal neuronal differentiation and then die when cultured in isolation in defined, serum-free medium (LCM). However, if purified progenitors are plated onto feeder layers of mitotically inactivated stromal fibroblasts, small numbers of neuronal colonies develop and are visible by 7 days in culture. The frequency at which neuronal colonies develop in culture (~1 per 3600 purified progenitors), plus the fact that these colonies contain undifferentiated cells which continue to incorporate ³H-TdR and generate new ORNs for at least 7 days *in vitro*, together suggest that the cell that gives rise to neuronal colonies may be the neuronal stem cell of the OE (Mumm *et al.*, 1996). Because this putative neuronal stem cell of the OE has not been identified directly, rather its activity is deduced from numbers of neuronal colonies that develop in culture, this cell has been called the 'neuronal colony-forming cell' or 'neuronal colony-forming unit (CFU)', by analogy to

CFUs thought to be indicative of stem cells in haematopoietic development (Mumm *et al.*, 1996).

Studies of neuronal CFUs from the OE have also provided insight into how cell interactions within the OE may regulate neurogenesis. *In vivo*, if one olfactory bulb (the synaptic target of ORNs) is removed from the brain of an adult rodent, nearly all ORNs in the OE on the side that innervated that bulb die (Holcomb *et al.*, 1995). As ORNs die and the OE degenerates (decreases in thickness), neuronal progenitor cells, which lie underneath the dying neurons within the OE, proliferate and replace the lost ORNs (Gordon *et al.*, 1995). These observations suggest that neuronal progenitors *in vivo* – which are in close physical proximity to ORNs in the cell layers above them – can ‘read’ the number of differentiated neurons in their immediate environment and regulate their production of new neurons accordingly. Studies of OE neuronal colony-forming cells appear to have provided an *in vitro* correlate of this phenomenon. When purified neuronal progenitor cells are plated onto stromal feeder layers in the presence of a large excess of differentiated ORNs, the number of neuronal CFUs that develops is dramatically reduced from that observed in control cultures, or cultures to which a large excess of a different cell type has been added (Mumm *et al.*, 1996). This result suggests that differentiated ORNs produce a signal that feeds back to inhibit production of new neurons by their own progenitors, and could explain the surge in neurogenesis in the OE *in vivo* that occurs as a consequence of ORN death following bulbectomy. This increase in neurogenesis could be due to *loss* of an inhibitory signal, normally produced by living ORNs. By identifying the molecule(s) responsible for the ORN-derived inhibitory signal and characterizing the action of this signal on neuronal progenitors of the OE, it may be possible to gain insights into growth regulatory signals that are of fundamental importance for the regulation of neurogenesis in the developing mammalian nervous system, and to determine if persistence of these same signals also accounts for the fact that neurons cannot be regenerated when their numbers have been reduced by developmental defects, injury, or aging.

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