

USE OF BROMODEOXYURIDINE IMMUNOHISTOCHEMISTRY FOR THE STUDY OF CYTOGENESIS AND NEUROCHEMICAL AND CELLULAR DIFFERENTIATION IN THE MARSUPIAL BRAIN AND RETINA

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ABSTRACT

Determining the birthdate of neurons and glia in relation to the time period of neurochemical and cellular differentiation are necessary for understanding early developmental events in the central nervous system (CNS). Bromodeoxyuridine (BrdU), a thymidine analog has recently become an alternative to the tritiated thymidine autoradiography for the study of cytogenesis in the CNS. Our laboratory utilizes the Brazilian opossum, *Monodelphis domestica* to study the ontogeny of neuropeptide systems and the development of the visual system in the mammalian brain. Opossum pups are born in an extremely immature state before neurogenesis and morphogenesis are completed and provide an excellent experimental model for developmental studies. To this end, we have developed single, double, and triple label BrdU immunohistochemical procedures for simultaneous study of cytogenesis and cellular differentiation in the opossum brain and retina. These methods are simple, rapid, and very effective and could be widely applied for understanding developmental events in the CNS.

INTRODUCTION

The cytogenesis and differentiation of the mammalian central nervous system (CNS) begins with the proliferation of the neuroepithelium lining the ventricular system of the brain and spinal cord. The postmitotic neurons then migrate from the ependyma to their destined locations (Boulder Committee, 1970). The birthdating of neurons and glia in relation to the time period for expression of neurochemical and cellular phenotypes is an important issue in order to understand the functional relationships of neurochemicals and cellular differentiation. Although, many investigations have studied neurogenesis of the central nervous system in a variety of mammalian species and have provided a detailed account of the time of completion of neurogenesis of most brain regions (Ifft, 1972; Shimada and Nakamura, 1973; Altman and Bayer, 1978; Karim and Slopper, 1980; Van Erden and Rakic, 1994). However, the possible existence of time differences in the formation of neurons and glia which express different chemical and cellular phenotypes has not been fully investigated. Further, it is not known if the neurons that express a specific neurochemical phenotype and have a specific function may have

different time periods of proliferation in contrast to the cells in the surrounding areas.

Our laboratory utilizes the Brazilian gray short-tailed opossum (*Monodelphis domestica*) as an experimental model to study the ontogeny of neuropeptide systems and to examine the development of the visual system in the mammalian brain. The Brazilian opossum is a small pouchless marsupial, whose young are born in an extremely immature and sexual undifferentiated state and have a protracted period of postnatal cytogenesis (Kuehl-Kovarik *et al.*, 1995). Thus, the Brazilian opossum serves as an exceptionally useful experimental model for developmental studies (Iqbal *et al.*, 1995; Kuehl-Kovarik *et al.*, 1995).

Bromodeoxyuridine (BrdU) is a thymidine analog which has recently become an alternative to tritiated thymidine for the study of developmental events in the CNS (Nowakowski *et al.*, 1989; Takahashi *et al.*, 1993). Following exogenous administration, BrdU is incorporated into the DNA during the synthesizing phase (S phase) of the cell cycle. Like tritiated thymidine, it remains in the postmitotic cells permanently and the BrdU containing cells can then be detected by using an anti BrdU antibody directed against single stranded DNA and immunohistochemical

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protocols (Gray, 1985). We have developed single, double, and triple label BrdU immunohistochemistry protocols for simultaneous study of cytogenesis and cellular differentiation, which will be of a significant interest for neurobiologist studying the birdating of neurons and glia in relation to their neurochemical and cellular phenotypes. In the present report, we describe the protocols in detail for single, double, and triple label BrdU immunohistochemistry.

MATERIALS AND METHODS

Developing Brazilian opossum pups were obtained from a colony maintained at Iowa State University. The care and procedures used in this study were in accordance with those approved by the Iowa State University Committee on Animal Care.

On each of the days between postnatal day 1 and 15 (PN 1-15; day 1 defined as day of birth), a different litter of opossum pups was injected with BrdU solution. Individual pups were injected while attached to the mother's nipple. Each pup was given a subcutaneous injection of 20 μ l of BrdU solution (Sigma; 1 mg/ml), dissolved in 0.9 per cent saline along the dorsal midline. All animals in each litter were weighed, examined for the presence of any abnormalities, and killed at 60 PN by over-etherization. Some of the animals were killed at 30 PN for the study of cytogenesis of the retina. Following over-etherization, the animals were perfused transcidentally with 0.9 per cent saline followed by Zamboni's fixative solution for 10 minutes (Iqbal *et al.*, 1995). Brains and eyes were removed from the calvaria and postfixed for 48 hours at room temperature in Zamboni's fixative solution. Subsequently brains and eyes were sunk in 30 per cent sucrose solution, and 20 μ m thick coronal sections were cut on a cryostat (Reichert Instruments) at -20°C. Tissue sections were thaw mounted onto poly-L-lysine coated slides, kept on a slide warmer at 37°C overnight, and stored at 4°C until immunohistochemistry was conducted.

Immunohistochemical procedures

The protocol utilized for single label BrdU immunohistochemistry was a modification of that which has been reported earlier (Millar and Nowakowski, 1988).

Pretreatment and DNA denaturation

1. Rinse tissue sections on slides with 50 mM potassium phosphate buffered saline (KPBS, pH 7.4) two times (2X) for 5 minutes each at room temperature.
2. Incubate the tissue sections in trypsin presoak for 30 minutes at 37°C in an oven. The trypsin presoak solution is made as follows: (0.18 g of trypsin; Sigma Type III, Bovine and 0.18 g CaCl₂,

dissolved in 300 ml of KPBS).

3. Wash 2X for 5 minutes each with KPBS.
4. Incubate tissue sections in 0.1N HCl (ice cold) for 10 minutes. This is made by adding 2.78 ml of concentrated (37%) HCl to 300 ml of distilled water. (The HCl solution should be chilled in the freezer towards the end of the trypsin presoak). The slides should be placed in the ice cold HCl in the freezer throughout the 10 minute incubation period.
5. Incubate in 2N HCl at 37°C in an oven for 30 minutes. The solution is made by adding 50 ml of concentrated HCl to 250 ml of KPBS.
6. The tissue sections are then neutralized in basic KPBS (pH 8.5) for 10 minutes at room temperature.
7. Rinse the tissue with KPBS 2X for 5 minutes each and follow the procedure for the single label BrdU immunohistochemistry.

Single label BrdU immunohistochemistry

8. Incubate the tissue in 0.3 per cent hydrogen peroxide solution to remove endogenous peroxidase activity. This is made by adding 30 ml of 3 per cent H₂O₂ to 270 ml of KPBS.
9. Incubate the tissue in normal horse serum used as a blocking agent (Vector, 1:67) for 2 hours at room temperature in a humidified chamber.
10. Incubate the tissue in BrdU antisera (1:200, Mouse monoclonal IgG, Dako) overnight at room temperature in a humidified chamber.

Second day

11. Wash the tissue 10X for 10 minutes each with KPBS containing 0.02 per cent Triton X-100 (Sigma).
12. Expose the tissue sections to horse anti-mouse IgG (Vector; 1:200) for 2 hours at room temperature in a humidified chamber.
13. Rinse the sections with KPBS 4X for 10 minutes each.
14. React the tissue sections with avidin-biotin complex (ABC; Vector Elite Kit, 1:50) at room temperature for one hour.
15. Rinse the tissue sections with KPBS 2X for 5 minutes each and 2X for 5 minutes each in 0.1 M sodium acetate.
16. React the tissue sections with a substrate composed of 0.04 per cent 3', 3' diaminobenzidine tetrahydrochloride (DAB; Sigma), 2.5 per cent nickel sulfate (Fisher Scientific) and 0.01 per cent hydrogen peroxide, in 0.1 M sodium acetate solution for 5-6 minutes.
17. Terminate the reaction by rinsing the tissue sections 2X for 5 minutes each with normal saline solution.

Finally, the sections are dehydrated in graded alcohols, cleared in xylene, and coverslipped with Permount mounting media (Fisher Scientific) and analyzed with light microscope. This procedure yields dark purple nuclear staining.

Double label BrdU immunohistochemistry

For double label immunostaining, sections are first processed for BrdU single label immunohistochemistry using ABC and DAB-nickel procedures as described above using steps 1-17. Subsequently, the tissue sections are processed for double label immunostaining.

18. Rinse the sections with KPBS 2X for 5 minutes each.
19. Incubate in 0.3 per cent hydrogen peroxide solution for 20-30 minutes to remove peroxidase activity due to the BrdU single label immunostaining.
20. Block in normal serum (normal goat serum, Vector; 1:67) for 2 hours at room temperature in a humidified chamber.
21. Incubate the sections in the primary antisera overnight. For brain, we incubated the tissue with arginine vasopressin antisera (1:5000; Rabbit polyclonal, Miles Scientific) or with oxytocin antisera (1:3000; Rabbit polyclonal, Peninsula Laboratories) and for retina we incubated the tissue with glial fibrillary acidic protein (GFAP) antisera (1:1000, Rabbit polyclonal, sigma) for 20 hours at room temperature in a humidified chamber.
22. Tissue sections are washed with KPBS 8X for 10 minutes each.
23. Incubate the tissue sections in biotinylated goat anti-rabbit IgG (Vector; 1:800) for 2 hours at room temperature.
24. Wash sections with KPBS 4X for 10 minutes each and incubate in avidin-biotin complex (ABC; Vector, 1:200) for one hour at room temperature.
25. Rinse the tissue sections in KPBS 2X for 5 minutes each and 2X for 5 minutes each with 0.1M sodium acetate.
26. After rinsing, the tissue sections are reacted with DAB substrate (without added nickel sulfate) for 5-6 minutes. The reaction is then terminated by placing the tissue sections into normal saline solution 2X for 5 minutes each.

Finally, the sections are dehydrated in graded alcohols, cleared in xylene and coverslipped with Permount mounting media. Tissue sections are analyzed using a light microscope. Using this procedure we were able to visualize purple black (BrdU labelled nuclei) and brown (AVP, OT or GFAP-like labelled cytoplasm) reaction products simultaneously.

Triple label immunostaining

For triple labelling, BrdU single label immunohistochemistry is coupled with immunofluorescence immunohistochemistry. The tissue sections are processed for BrdU single label immunostaining as described above (steps 1-17) and the following steps are then carried out.

1. After the DAB reaction, wash the tissue sections with saline solution 2X for 5 minutes each and process the tissue sections in the following order for simultaneous localization of two cytoplasmic antigens.
2. Incubate the tissue sections in the natural blocking serum (our secondary antibodies are made in goat, thus we used normal goat serum for blocking step) for 2 hours at room temperature in the humidified chamber.
3. Incubate the tissue sections in the first "cytoplasmic" primary antiserum overnight in the humidified chamber at room temperature. (We incubated our tissue in OT primary antisera; 1:500, Rabbit polyclonal, Peninsula Labs.).
4. Wash tissue section with KPBS 6X for 10 minutes each.
5. Incubate in biotinylated goat anti-rabbit IgG (Vector; 1:200) for 2 hours in a humidified chamber at room temperature.
6. Wash the tissue sections with KPBS 3X for 10 minutes each.
7. Incubate the tissue sections in Texas red avidin-D conjugate (Vector; 25 µg/ml of sodium bicarbonate buffer) for one and a half hours at room temperature in a humidified chamber.
8. Wash the tissue sections with KPBS 3X for 10 minutes each.
9. Block the tissue sections in the normal blocking serum.
10. Incubate the sections in the second 'cytoplasmic' primary antibody (we used AVP primary antisera, generated in guinea pig; 1:500, Peninsula Labs.) overnight at room temperature in humidified chamber. The primary antibody for the second antigen must be generated in a different species than that for the first.
11. Wash tissue sections with KPBS 6X for 10 minutes each.
12. Incubate the tissue sections with fluorescein conjugated goat anti-guinea pig IgG (Vector; 1:200) for 2 hours at room temperature in a humidified chamber.
13. Wash the tissue sections with KPBS 2X for 10 minutes each.
14. Coverslip with glycerol (Dako).
15. Tissue sections are viewed with a fluorescence microscope using appropriate filter cubes.

Using this procedure we were able to visualize BrdU (purple black nuclei), OT (bright red), and AVP (bright green) labelling simultaneously by switching between bright field and FITC and Texas red fluorescence filters.

Immunohistochemical control procedures

Negative and preabsorption controls are generated for every single, double, and triple label run as has been reported earlier (Iqbal *et al.*, 1995).

Analysis of tissue

Tissue sections processed for BrdU single label and double label immunohistochemistry were analyzed with a light microscope. Tissue sections processed for triple label immunohistochemistry were analyzed with a Zeiss Axiophot microscope equipped with a mercury light source and appropriate fluorescence filter cubes. Double label and triple labelled tissue sections were observed by switching between the filter systems and bright field illumination. Kodak TMax 400 film was utilized for black and white photomicrography of the tissue sections processed for single labelling. Tissue sections processed for double labelling were photomicrographed using Kodak Elite Ektachrome 100. Photomicrographs (double and/or triple exposure) of tissue sections processed for triple labelling were taken using Kodak Elite Ektachrome 400 color films using a Nikon Microphot XA equipped with automatic exposure meter.

RESULTS AND DISCUSSION

Analysis of tissue sections processed for single label BrdU immunohistochemistry demonstrated that the injection of BrdU solution subcutaneously into developing opossum pups effectively labelled the dividing cells throughout the brain and retina. Further, we did not observe any abnormality in the body weight, growth of the animals, or in brain or retinal morphology.

Examination of the tissue using bright field illumination revealed dark purple BrdU labelled nuclear staining of cells in the brain (Fig. 1A) and retina (Fig. 1B). No BrdU labelling was seen in a given region after completion of cytogenesis, except for a few small glial-like cells in the ependymal layer of the ventricles in the brain and along the inner retina.

Several double labelled cells (BrdU-AVP or BrdU-OT) containing dark purple nuclei (BrdU) and brown cytoplasmic immunostaining (AVP or OT) were clearly demonstrable in the tissue sections of the brain processed using sequential BrdU double label immunohistochemical protocols (Fig. 1 A).

In the retina, cytogenesis and cellular differentiation begins centrally and proceeds towards the periphery. In order to correlate cytogenesis with cellular differentiation in the retina, we have used double label immunohistochemistry for BrdU and GFAP, a marker for astrocytes in the opossum retina. Figure 1 B illustrates an example of a BrdU-GFAP double labelled astrocyte within the optic nerve head region. Utilizing DAB-nickel and DAB without nickel yielded a permanent record of double labelled cells which does not fade like immunofluorescence double labelling, and facilitated simultaneous visualization of double labelled cells without changing the optics or the light sources of the microscope.

Analysis of the tissue sections processed for BrdU-OT-AVP triple label immunohistochemistry revealed several double and/or triple labelled cells (Fig. 1 C,D). Comparison of the brain tissue processed singly for AVP or OT immunohistochemistry for another study with that processed for BrdU-AVP or BrdU-OT did not reveal any significant difference in the immunostaining patterns.

Bromodeoxyuridine has been used by various laboratories to study cell proliferation, migration, time of origin of neurons in the central nervous system, cytokinetic behaviour in the cerebral ventricles, and determination of cell cycles in the fetal mouse brain (Millar and Nowakowski, 1988; Nowakowski *et al.*, 1989; Takahashi *et al.*, 1993). Use of BrdU to study the birth dates and neurochemical phenotypes of neurons using simultaneous double label immunohistochemistry has also been reported (Beffo *et al.*, 1992). We have also reported the postnatal development of the paraventricular and supraoptic nuclei in the Brazilian opossum brain using BrdU single and simultaneous double and/or triple labelling immunohistochemistry (Iqbal *et al.*, 1995).

Previously, Miller and Nowakowski (1988) compared the labelling patterns after BrdU and tritiated thymidine injection given separately or together and have reported comparable numbers of BrdU and tritiated thymidine labelled cells in the rat brain. Analysis of tissue from animals injected with tritiated thymidine for a different study in our laboratory (Larsen and Jacobson, 1986) also revealed a comparable timing for neurogenesis and numbers of BrdU or tritiated thymidine labelled cells in the opossum brain (Iqbal and Jacobson, unpublished results).

In addition to single and double labelling immunostaining, we have developed a sequential triple label BrdU immunohistochemical protocol. Use of these procedures in our laboratory have demonstrated that the protocol is quite effective for the simultaneous study of

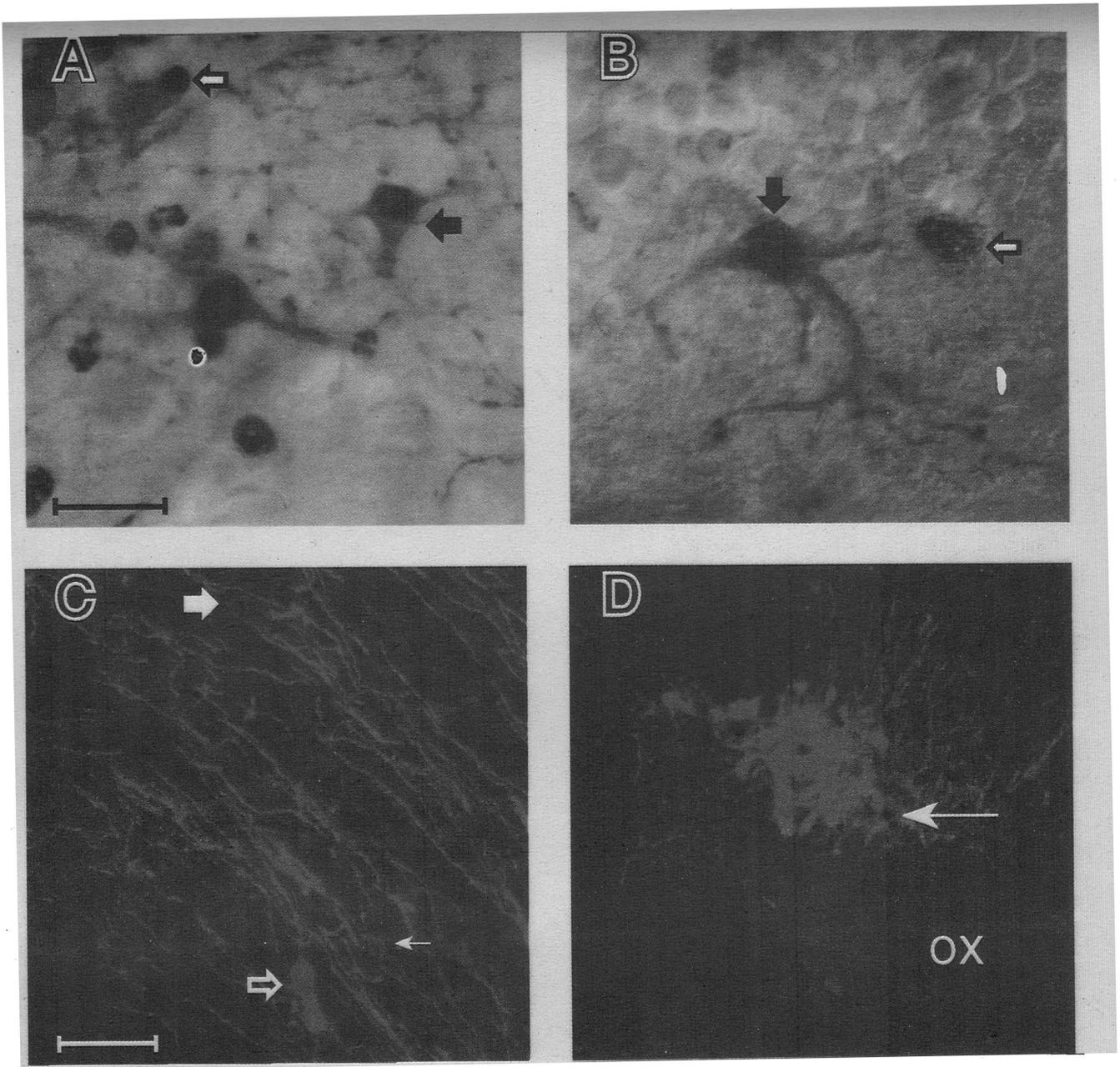


Fig. 1: Photomicrographs of tissue sections processed for double and triple label immunohistochemistry. Photomicrographs A and B demonstrate BrdU single labelled cells (black nuclear staining, black open arrows). The black solid arrow in A points to a BrdU-AVP double labelled cell (BrdU-black nuclear staining, AVP-brown cytoplasmic staining) in the paraventricular hypothalamic nucleus of a 60 days postnatal (60 PN) opossum, which was injected with BrdU at 2 PN. The black solid arrow in B points to a BrdU-GFAP double labelled cell, an astrocyte (BrdU-black nuclear staining, GFAP-brown cytoplasmic staining) in the optic nerve head region of the retina of a 60 PN animal which was injected with BrdU at 14 PN. Photomicrograph C demonstrates a BrdU-AVP double labelled cell (BrdU-black nuclear staining, AVP-green fluorescence, white arrow); an oxytocin labelled cell (red fluorescence, white open arrow), and an interaction between an AVP immunoreactive cell and an OT immunoreactive fiber in the lateral hypothalamic area indicated with a small white arrow. Photomicrograph D demonstrates a triple labelled (BrdU-OT-AVP) cell indicated by the long white arrow in the supraoptic nucleus of the 60 PN opossum which was injected with BrdU at 2 PN. In A and C medial is towards the left side of the photomicrographs. Abbreviation: OX, optic chiasm. All photomicrographs are at the same magnification. Scale bar = 50 μ m.

neurogenesis and the time course of development of neuropeptide systems and cytochrome and cellular differentiation in the opossum CNS and retina (Stone *et al.*, 1994; Iqbal *et al.*, 1995). We believe that these multilabelled immunohistochemical protocols provide an extremely useful technique that can be effectively employed in other mammalian species for similar neurocytological studies. In addition to studying the origin and migration of neurons expressing specific neurochemicals, we believe that this type of immunohistochemical analysis can be coupled with additional studies to determine neuron-glia interactions, as well as tract tracing techniques, to determine the time course of development of axonal projections of different neuronal populations expressing specific neurochemical phenotypes. In addition to qualitative analysis, BrdU immunohistochemistry can easily be used for quantitative analysis using light microscopy and an image analysis system (Iqbal *et al.*, 1995).

Denaturation of the DNA with enzymatic and HCl treatments of the tissue are essential for BrdU immunohistochemistry, since the BrdU antibody is generated against the single stranded DNA. We did not observe any significant effects of denaturation on AVP and/or OT immunoreactivity. However, these procedures may affect the immunoreactivity of other neuropeptides, a problem which might be overcome by using higher concentrations of the primary antibody, incubating the tissue for longer time periods in the primary antibody, by omitting the enzymatic pretreatment, or trying a different enzyme such as pepsin or proteinase.

Further, for triple labelling immunohistochemistry the primary antisera for the second and third antigens must be generated in different species and should be tested for cross reactivity by preabsorption immunohistochemical control procedures.

In summary, we have developed a simple, rapid, and effective BrdU single, double, and triple labelling immunohistochemical protocol for simultaneous study of neuronal and glial birthdating and their differentiation of neurochemical and cellular phenotypes. These methods are also useful for quantitative analysis of histogenesis of the brain. Further, there are no radiation hazards as are associated with tritiated thymidine autoradiography. Based on our results we believe that this protocol could be of great significance for a wide range of developmental studies.

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