Journal of Neuroscience Methods 144 (2005) 197–201

JOURNAL OF NEUROSCIENCE METHODS

www.elsevier.com/locate/jneumeth

# RNA amplification of bromodeoxyuridine labeled newborn neurons in the monkey hippocampus

Scott E. Counts[a], Er-Yun Chen[a], Stephen D. Ginsberg[b], Jeffrey H. Kordower[a], Elliott J. Mufson[a,*]

[a] *Department of Neurological Sciences, Alla and Solomon Jesmer Chair in Aging, Rush University Medical Center, 1735 W. Harrison Street, Suite 300, Chicago, IL, USA*

[b] *Center for Dementia Research, Nathan Kline Institute, Departments of Psychiatry and Physiology & Neuroscience, New York University School of Medicine, Orangeburg, NY, USA*

Received 24 September 2004; received in revised form 4 November 2004; accepted 4 November 2004

**Abstract**

Neurogenesis has been demonstrated in the adult mammalian hippocampus by the immunohistochemical identification of cells co-labeled with the neuronal marker NeuN and bromodeoxyuridine (BrdU), a marker for DNA synthesis. Whether these newly born neurons exhibit a genetic signature similar to that of existing hippocampal cells remains unknown. Recent advances in single cell RNA amplification techniques provide a unique method for profiling the mRNA complement of cells developed during adult neurogenesis. Standard protocols for identifying BrdU-positive cells requires an acid denaturation step that may preclude the amplification of cellular RNA for expression analysis. We first tested whether the BrdU reaction product was visible in monkey hippocampal tissue following treatment with dilutions of HCl (2–0.2 M) or citric acid (1.0–0.1 M). BrdU-labeled cells were visible only in tissue sections treated with 2 M HCl. RNA amplification was not compromised in cells dual-labeled for BrdU and NeuN using the 2 M HCl acid denaturation step. These cells express mRNAs encoding a wide variety of functional protein subclasses including glutamate receptors. The present study demonstrates for the first time that BrdU immunohistochemisty is compatable with gene array technology in the primate hippocampus to evaluate subclasses of genes in newborn neurons.


*Keywords:* Neurogenesis; Hippocampus; Bromodeoxyuridine; RNA amplification; cDNA array; Primate

## 1. Introduction

Neurogenesis in the dentate gyrus of the hippocampus has been demonstrated across the lifespan of a variety of mammalian species including old world monkeys and humans (Altman and Das, 1965; Kaplan and Hinds, 1977; Eriksson et al., 1998; Gould et al., 1999; Kornack and Rakic, 1999). Adult-generated cells immunopositive for bromodeoxyuridine (BrdU), a marker for DNA synthesis and neuronal markers, such as NeuN, arise from hippocampal progenitor cells in the hilus or subgranular zone and migrate to the granule cell layer (GCL), where they differentiate into neurons (Cameron et al., 1993; Kuhn et al., 1996; Eriksson et al., 1998). These new neurons extend processes and are morphologically similar to mature granule cells, suggesting they may integrate into the GCL circuitry. Our laboratory has been interested in determining the genetic signature of newborn hippocampal neurons. One avenue toward characterizing the phenotype of newborn hippocampal neurons is to examine the complement of functional classes of mRNAs expressed by these cells using recently developed single cell RNA amplication techniques (Eberwine et al., 1992; Ginsberg and Che, 2002; Mufson et al., 2002; Che and Ginsberg, 2004). Single cell RNA amplification combined with cDNA array technology can be used to evaluate the relative expression levels of functional classes of genes involved in a variety of cellular mechanisms, including neurotransmitter metabolism and/or synaptic transmission, which might provide clues to neuronal maturity and functionality of these newly derived

* Corresponding author. Tel.: +1 312 563 3558; fax: +1 312 563 3571.
*E-mail address:* emufson@rush.edu (E.J. Mufson).

0165-0270/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jneumeth.2004.11.005

cells. However, methods used to label cells for BrdU incorporation involve a nucleic acid denaturation step using 2 M HCl that may compromise the structural integrity of cellular RNA, thus preventing enzymatic amplification of mRNA templates for gene expression analysis. For example, use of Bouin's solution (which contains picric acid) for brain tissue fixation has been demonstrated to compromise the ability to identify and extract RNA species (Ginsberg et al., 1997, 1998). The present report describes an experimental procedure that allows for the combined immunohistochemical identification of BrdU/NeuN double-labeled neurons with single cell RNA amplification coupled with custom-designed cDNA arrays for evaluating the genetic signature of newborn neurons in the primate hippocampus. This dual immunomolecular technique provides a novel approach for the examination of functional classes of genes expressed in newly generated neurons in the mammalian brain.

## 2. Materials and methods

### 2.1. BrdU treatments

Three young adult female cynomolgus monkeys (*Macaca fasciculari*) were used for this study. The animals were singly housed with a 12-h light/dark cycle. Purina monkey chow and water was available ad libitum. The study was performed in accordance with federal guidelines of proper animal care and with the approval of the Rush Institutional Animal Care and Use Committee. The monkeys were anesthetized with ketamine (20 mg/kg body weight, i.m.) and received BrdU (75 mg/kg, i.v.; Sigma, St. Louis, MO) dissolved in normal saline and warmed to 65 °C. Treatments were administered by catheterization every 24 h at ∼10:00 a.m. for four successive days. Animals were sacrificed 7 days after the last BrdU treatment for tissue collection.

### 2.2. Tissue preparation

Monkeys were pretreated with ketamine (20 mg/kg, i.m.) and then deeply anesthetized with sodium pentobarbital (20 mg/kg, i.v.). The monkeys were then injected with 1 ml of heparin (20,000 IU) into the left ventricle and perfused transcardially with 0.9% saline followed by fixation with a 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB, pH 7.4). Brains were removed from the calvaria, post-fixed overnight in the same fixative and cryoprotected through graded sucrose (10, 20 and 30%) in phosphate-buffered saline (PBS, pH 7.2) at 4 °C. Serial sections (40 μm, six series) throughout the brain were cut frozen on a sliding knife microtome and stored at −20 °C in cryoprotectant.

### 2.3. Double label immunohistochemistry for BrdU and NeuN

Double label immunoperoxidase procedures were employed to determine whether BrdU-positive cells co-expressed NeuN within the dentate gyrus of the hippocampus (Chen et al., 2000; Brown et al., 2003). All procedures were performed in RNase-free conditions to facilitate single cell RNA expression analysis. Sections were washed three times at ambient room temperature (RT) in Tris-buffered saline (TBS, pH 7.4) containing 0.05% Triton X-100 (TBS-Tx) and then incubated for 30 min in TBS containing 1% $H_2O_2$ to eliminate endogenous peroxidase activity. After three washes in TBS, sections were denatured with 2 M HCl at 37 °C for 30 min and then neutralized with 0.1 M borate buffer (pH 8.5) for 10 min. This step is performed to denature cellular DNA, thus allowing the BrdU antibody access to BrdU incorporated into the DNA during replication. However, 2 M HCl treatment might preclude subsequent RNA amplification procedures on BrdU-labeled neurons if cellular mRNA is denatured as well. Therefore, alternative denaturation steps using a range of lower HCl concentrations (1.5, 1.0, 0.5 and 0.2 M) or citric acid (1.0 and 0.1 M) were also performed in parallel experiments to test whether less stringent acid denaturation conditions would allow for BrdU visualization (see below). After washing with TBS-Tx, sections were blocked for 1 h in TBS-Tx containing 5% normal goat serum (NGS) and incubated overnight at room temperature (RT) with rat anti-BrdU antibody (1:500, Accurate, Westbury, MA) suspended in PBS/0.4% Tx/3% NGS. After six washes in TBS-Tx, sections were sequentially incubated at RT with biotinylated goat anti-rat IgG (Vector labs, Burlingame, CA; 1:200; 1 h) and the "Elite" avidin–biotin complex (ABC, Vector labs; 1:500; 75 min) separated by six washes in TBS-Tx. Sections were then reacted with 0.05% 3′,3-diaminobenzidine (DAB), 0.005% $H_2O_2$ and 2.5% nickel II sulfate, yielding a black reaction product for the BrdU-labeled cells. Following this reaction, the sections were processed for NeuN immunoreactivity. Sections were washed with TBS-Tx and blocked with TBS-Tx containing 5% normal horse serum (NHS) for 1 h at RT. Mouse anti-NeuN antibody (1:1000, Chemicon, CA) was applied overnight at RT in PB/0.4% Tx/3% NHS. After TBS-Tx washes, sections were sequentially incubated at RT with biotinylated horse anti-mouse IgG (Vector labs, 1:200; 1 h) ABC (Vector labs, 1:500; 75 min) separated by TBS-Tx washes. Sections were then reacted with 0.05% DAB and 0.005% $H_2O_2$ yielding a brown reaction product for the NeuN-labeled cells. Sections were mounted on gelatin-coated slides, allowed to dry at RT, and then stored in RNase-free PB at 4 °C. The black and brown reaction products allowed for an easily identifiable two-color reaction profile (Chen et al., 2000). GCL neurons of interest were microaspirated from the tissue within 1 week of storage in RNase-free PB.

### 2.4. Single cell RNA amplification procedures

The amplification of mRNA from GCL neurons (both NeuN single and BrdU/NeuN-double labeled cells) was performed using a new terminal continuation (TC) RNA amplification methodology (Ginsberg and Che, 2002;