Neuroscience 146 (2007) 108–122

DETERMINATION OF KEY ASPECTS OF PRECURSOR CELL PROLIFERATION, CELL CYCLE LENGTH AND KINETICS IN THE ADULT MOUSE SUBGRANULAR ZONE

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Abstract—Neurogenesis studies on the adult mouse hippocampal subgranular zone (SGZ) typically report increases or decreases in proliferation. However, key information is lacking about these proliferating SGZ precursors, from the fundamental—what dose of bromodeoxyuridine (BrdU) is appropriate for labeling all S phase cells?—to the detailed—what are the kinetics of BrdU-labeled cells and their progeny? To address these questions, adult C57BL/6J mice were injected with BrdU and BrdU-immunoreactive (IR) cells were quantified. Initial experiments with a range of BrdU doses (25–500 mg/kg) suggested that 150 mg/kg labels all actively dividing precursors in the mouse SGZ. Experiments using a saturating dose of BrdU suggested BrdU bioavailability is less than 15 min, notably shorter than in the developing mouse brain. We next explored precursor division and maturation by tracking the number of BrdU-IR cells and colabeling of BrdU with other cell cycle proteins from 15 min to 30 days after BrdU. We found that BrdU and the Gap2 and mitosis (G_2/M) phase protein pHisH3 maximally colocalized 8 h after BrdU, indicating that the mouse SGZ precursor cell cycle length is 14 h. In addition, triple labeling with BrdU and proliferating cell nuclear antigen (PCNA) and Ki-67 showed that BrdU-IR precursors and/or their progeny express these endogenous cell cycle proteins up to 4 days after BrdU injection. However, the proportion of BrdU/Ki-67-IR cells declined at a greater rate than the proportion of BrdU/PCNA-IR cells. This suggests that PCNA protein is detectable long after cell cycle exit, and that reliance on PCNA may overestimate the length of time a cell remains in the cell cycle. These findings will be critical for future studies examining the regulation of SGZ precursor kinetics in adult mice, and hopefully will encourage the field to move beyond counting BrdU-IR cells to a more mechanistic analysis of adult neurogenesis.

Key words: BrdU, PCNA, pHisH3, Ki-67, mitosis, neurogenesis.

Adult hippocampal neurogenesis is increasingly appreciated as a process, not a time point (Kempermann et al., 2004). It begins with precursor proliferation, progresses through neuronal differentiation and survival, and culminates in integration of neurons into hippocampal circuitry. While evidence suggests that each step of this process can be regulated, it is clear that many manipulations that alter adult hippocampal neurogenesis do so by influencing the proliferation of precursors in the subgranular zone (SGZ). This distinction between effects on proliferation and survival underscores the importance of understanding key technical aspects of how cells in the adult mouse SGZ divide. Additional information about how mouse SGZ cells divide and how long they reside in the cell cycle would allow for a more mechanistic exploration of regulation of adult neurogenesis.

The first key piece of information that is needed is the optimal dose of the exogenous marker, bromodeoxyuridine (BrdU), for labeling all cells in S phase of the cell cycle. BrdU doses given to adult mice vary from multiple injections of 50 mg/kg to a single injection of 150 mg/kg (Kempermann et al., 1998; Mandyam et al., 2004), and therefore likely label different cohorts of S phase cells. Identification of the S phase saturating dose of BrdU is a key first step in precise evaluation of the mechanisms underlying regulation of proliferation, and will foster comparison of results across laboratories. An elegant study by Cameron and McKay (2001) showed that in the adult rat, a single high dose of BrdU saturated the S phase population without causing overt damage to the labeled cells (see Tables 1 and 2 in Cameron and McKay, 2001). However, such information is lacking for the mouse. Given that transgenic mice are increasingly assessed for alterations in proliferation and neurogenesis, and that SGZ precursors appear to be distinct in rat versus mouse (see below), determination of which dose of BrdU is appropriate for mouse studies is needed.

A second piece of information that is needed involves the cell cycle of mouse SGZ precursors. Estimates of the cell cycle and its components in the rat and mouse SGZ are very different (rat vs. mouse: length of cell cycle, 24.7 h vs. 12–14 h; length of S phase, 9.5 h vs. 6–7.6 h; percent of cell cycle devoted to S phase, 38% vs. 54–63%; percent of cell cycle devoted to Gap2 and mitosis (G_2/M), 18% vs. 32–38%; Cameron and McKay, 2001; Hayes and Nowakowski, 2002; Burns and Kuan, 2005). This fundamental information has been used to explore key technical details in the adult rat SGZ, such as following the fate and kinetics of several generations of BrdU-labeled cells and their prog-

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Abbreviations: AAALAC, Assessment and Accreditation of Laboratory Animal Care International; BrdU, bromodeoxyuridine; DAB, diaminobenzidine; G_2/M, Gap2 and mitosis; Hil, hilus; IHC, immunohistochemistry; IR, immunoreactive; Mol, molecular layer; oGCL, outer granule cell layer; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; pHisH3, phosphorylated histone H3; SGZ, subgranular zone.

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doi:10.1016/j.neuroscience.2006.12.064

eny after BrdU injection (Dayer et al., 2003). Such critical information is still needed for the mouse, even considering Hayes and Nowakowski's (2002) groundbreaking work in identifying other cell cycle parameters in the adult mouse SGZ. Such technical details of the mouse SGZ precursors will help us better utilize transgenic mice that are currently available to mark cells at different stages of cell division (Sawamoto et al., 2001; Overstreet et al., 2004), therefore allowing us to uncover cellular mechanisms in regulation of adult neurogenesis.

A final piece of information needed is how endogenous cell cycle proteins compare in their ability to provide insight into SGZ precursor proliferation. In studying adult neurogenesis, it is common to label and visualize precursors with exogenous S phase markers, such as BrdU (Miller and Nowakowski, 1988; Cameron and Gould, 1996). Alternatively, expression of endogenous cell cycle markers can be used to detect dividing precursors. Proliferating cell nuclear antigen (PCNA) and Ki-67 have long been used to assess regulation of neurogenesis in tissue where labeling with BrdU is not feasible or untenable, such as in natural populations and human postmortem tissue (Celis et al., 1986; Bacchi and Gown, 1993; Brown et al., 2003a; Curtis et al., 2003; Dayer et al., 2003; Wharton et al., 2005; Reif et al., 2006; Eisch and Mandyam, in press). Many studies refer to PCNA or Ki-67 as "endogenous cell cycle markers" and use them almost interchangeably as markers of dividing cells (Kee et al., 2002; Gil et al., 2005; He et al., 2005). However, review of the literature suggests that PCNA and Ki-67 have distinct characteristics that should be considered prior to using these markers for studies of adult hippocampal neurogenesis. For example, while Ki-67 expression indicates proliferation, PCNA expression can indicate proliferation, DNA repair, or cell death (Pandey and Wang, 1995). In addition, biochemical analyses indicate the half-life of PCNA is 20 times longer than the half-life of Ki-67 (Khoshyomn et al., 1993; Karamitopoulou et al., 1994; Lopez-Girona et al., 1995). Therefore, PCNA protein expression may remain detectable either long after cell cycle exit or may be reflective of cell death, thus the use of PCNA as a marker of proliferation may overestimate the number of cells in the cell cycle. *In vitro* data also suggest that PCNA and Ki-67 are not equally expressed in all cell cycle phases (Gerdes et al., 1984; Celis and Celis, 1985; Takahashi and Caviness, 1993; Kawabe et al., 2002; Eisch and Mandyam, 2004). In sum, while PCNA and Ki-67 are often used to label and study the regulation and cell cycle kinetics of proliferating cells, a detailed comparison of the strengths and limitations of these endogenous markers for adult neurogenesis studies is warranted.

Here we probe for answers to these questions about SGZ precursors by qualitatively and quantitatively examining BrdU-immunoreactive (IR) cells in the adult mouse SGZ at multiple time points after BrdU. We first assess the dose of BrdU sufficient to label all S phase cells in the adult mouse SGZ, and we explore whether a higher dose of BrdU labels more cells merely because it has a longer bioavailability. We explore how long BrdU-labeled daughter cells are added to the cell cycle in the adult mouse SGZ, quantifying BrdU-IR cells and clusters as well as colabeling with endogenous cell cycle proteins. We also specifically evaluate the utility of endogenous cell cycle proteins PCNA and Ki-67 to reveal information about SGZ precursors. Finally, we compare cell cycle kinetic data gleaned from using endogenous cell cycle proteins to previous estimations of cell cycle kinetics in the embryo, in other brain regions, and in other species.

EXPERIMENTAL PROCEDURES

Animals

Adult, male C57BL/6J mice (initial weight 23–27 g, 9–11 weeks old; Jackson Laboratories, Bar Harbor, ME, USA) were used. Mice were group housed (maximum five/cage) in a facility approved by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC) at the University of Texas Southwestern Medical Center, with a 12-h light/dark cycle and with free access to food and water. Mice were acclimated to vivarium conditions for at least 1 week prior to experimentation. All experiments conformed to guidelines of both the UT Southwestern Institutional Animal Care and Use Committee and AAALAC. The investigators took all steps to minimize the number of mice used for these experiments, and to minimize animal suffering.

BrdU injections and tissue preparation

For the BrdU dose response studies, mice received BrdU (Boehringer Mannheim, GmBH, Mannheim, Germany; dissolved in 0.9% saline and 0.007 N NaOH; one injection of 25–500 mg/kg given in equal volumes i.p.; $n=2–4$ per dose) to label dividing cells and were killed 2 h post-injection. For the BrdU time course study, mice received BrdU (one injection of 150 mg/kg i.p.; $n=4–6$ for each time point) to label dividing cells and were killed at various time points post-injection to examine proliferation (0.25, 2, 8, 15, and 24 h), differentiation (48 and 96 h, or 2 and 4 days), and survival (240 and 720 h, or 10 and 30 days). All mice were anesthetized with chloral hydrate prior to intracardial perfusion with chilled phosphate-buffered saline (1× PBS; 5 min, flow rate of 7 ml/min) and 4% paraformaldehyde in PBS (pH 7.4, 15 min). The brains were postfixed overnight at 4 °C with 4% paraformaldehyde and stored in 30% sucrose solution. Brains were cut through the hippocampus (bregma −0.82 to −4.24; Paxinos and Franklin, 2001) at 30 μm in the coronal plane on a freezing microtome as described previously (Eisch et al., 2000). Sections were stored at 4 °C in 0.1% NaN_3 in PBS until processed for immunohistochemistry (IHC).

Antibodies

The following primary antibodies were used for IHC. Rat monoclonal anti-BrdU (cat. # OBT0030; clone BU1/75-ICR1; Accurate, Westbury, NY, USA; 1:100) was raised against BrdU. Staining was not seen in animals that did not receive BrdU, and the pattern of staining was similar to that previously reported (Eisch et al., 2000; Mandyam et al., 2004). Rabbit polyclonal anti-phosphorylated histone H3 (pHisH3) (cat. # sc-8656; Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:50) was raised against amino acid sequence containing phosphorylated Ser 10 of histone H3 of human origin. This antiserum stains a single 20 kDa band on Western blot (Ng et al., 2004). Via IHC, the pattern of staining was similar to that previously reported (Mandyam et al., 2004), with cells presenting a diverse nuclei morphology reminiscent of pro-, meta-, ana-, and telophase. Mouse monoclonal anti-PCNA (cat. # MAB4078; clone 19A2; Chemicon International, Temecula, CA, USA; 1:4000) was raised against whole PCNA of human origin. This antiserum stains a single 36 kDa band on Western blot