

# Adult Neurogenesis Produces a Large Pool of New Granule Cells in the Dentate Gyrus

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## ABSTRACT

Knowing the rate of addition of new granule cells to the adult dentate gyrus is critical to understanding the function of adult neurogenesis. Despite the large number of studies of neurogenesis in the adult dentate gyrus, basic questions about the magnitude of this phenomenon have never been addressed. The S-phase marker bromodeoxyuridine (BrdU) has been extensively used in recent studies of adult neurogenesis, but it has been carefully tested only in the embryonic brain. Here, we show that a high dose of BrdU (300 mg/kg) is a specific, quantitative, and nontoxic marker of dividing cells in the adult rat dentate gyrus, whereas lower doses label only a fraction of the S-phase cells. By using this high dose of BrdU along with a second S-phase marker, [<sup>3</sup>H]thymidine, we found that young adult rats have 9,400 dividing cells proliferating with a cell cycle time of 25 hours, which would generate 9,000 new cells each day, or more than 250,000 per month. Within 5–12 days of BrdU injection, a substantial pool of immature granule neurons, 50% of all BrdU-labeled cells in the dentate gyrus, could be identified with neuron-specific antibodies TuJ1 and TUC-4. This number of new granule neurons generated each month is 6% of the total size of the granule cell population and 30–60% of the size of the afferent and efferent populations (West et al. [1991] *Anat Rec* 231:482–497; Mulders et al. [1997] *J Comp Neurol* 385:83–94). The large number of the adult-generated granule cells supports the idea that these new neurons play an important role in hippocampal function. *J. Comp. Neurol.* 435:406–417, 2001. Published 2001 Wiley-Liss, Inc.†

**Indexing terms:** bromodeoxyuridine; thymidine; cell cycle; mitosis; hippocampus; neurons

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New granule neurons are produced throughout adulthood in the dentate gyrus of mammals from rodents to humans (Cameron and McKay, 1998; Eriksson et al., 1998; Gould et al., 1999a). Although the existence of this phenomenon has gained widespread acceptance, there are still questions about its importance. The reported numbers of dividing cells in the adult dentate gyrus are often relatively small, suggesting that new neurons may not have much impact on hippocampal function. However, the actual rate of cell birth in the adult dentate gyrus has never been calculated; neither the total number nor the cell cycle time of dividing precursors in the adult dentate gyrus is known.

The development of 5-bromo-2'-deoxyuridine (BrdU) immunohistochemistry to identify S-phase cells in the brain (Miller and Nowakowski, 1988) was an important advance over [<sup>3</sup>H]thymidine autoradiography; this new method detects labeled cells throughout the relatively thick tissue sections required by stereologic techniques for

determining the total number of S-phase cells within a brain region (West et al., 1991). However, BrdU also has some drawbacks. First, it may not be absolutely specific for dividing cells, i.e., BrdU, like [<sup>3</sup>H]thymidine, could potentially label neurons that are repairing small amounts of DNA in addition to cells replicating their complete genome (Korr and Schultze, 1989; Selden et al., 1993; Schmitz et al., 1999). Additionally, BrdU may act as a mutagen in cells that incorporate it; high doses of BrdU have been shown to have adverse effects on embryonic and neonatal rats (Bannigan, 1985; Nagao et al., 1998; Kolb et al., 1999). To minimize these potential problems, investi-

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Received 22 November 2000; Revised 12 January 2001; Accepted 21 February 2001

gators aim to use the lowest dose of BrdU that results in visible labeling. An intraperitoneal (i.p.) injection of 50 mg/kg of BrdU is the lowest dose that labels every cell in the S-phase region of the cortical ventricular zone of embryonic mice and has no apparent toxic effects (Miller and Nowakowski, 1988; Takahashi et al., 1992). This same dose has been used for most studies of adult neurogenesis (Bengzon et al., 1997; Kempermann et al., 1998; Kornack and Rakic, 1999; van Praag et al., 1999). However, because of the development of the blood-brain barrier around the time of parturition, a dose suitable for embryonic studies may be too low for experiments in adults.

In this study, we gave different doses of BrdU to adult rats and counted BrdU-labeled cells in the dentate gyrus to determine the optimal dose for labeling granule cell progenitors. To determine whether there are adverse effects of these doses of BrdU in the adult dentate gyrus, we examined the fate of BrdU-labeled cells after different survival periods. We then determined the total number of dividing precursors in the adult rat dentate gyrus and calculated their cell cycle time to estimate the number of new cells produced in this region each day and used neuronal markers to determine what proportion of the new cells become neurons.

## MATERIALS AND METHODS

### Animal treatments

For all experiments, male rats were purchased from Taconic at least 5 days before treatment began, group-housed, and provided with unlimited access to food and water. All BrdU injections were i.p. All rats were deeply anesthetized with isoflurane inhalation followed by transcardiac perfusion with 4% paraformaldehyde (pH 7.4). Animal treatments conformed to NIH guidelines and were approved by the NINDS Institutional Animal Care and Use Committee.

**Experiment 1: Low doses of BrdU.** Four different doses of BrdU were given to sexually mature rats (100 g, 5 weeks old; male rats reach sexual maturity by P35 according to Wray and Hoffman, 1986). Each rat received a single injection of BrdU (Boehringer-Mannheim, 5 mg/ml in 0.007 N NaOH/0.9% NaCl) at a dose of 25, 50, 100, or 300 mg/kg ( $n = 6$  for each dose). The 50 and 100 mg/kg doses were chosen because they are the most commonly used in recent studies. Three rats in each dose group were perfused 24 hours after BrdU administration; this time point was chosen over a shorter time point to minimize possible effects of interanimal differences in survival time after injection. The remaining three rats in each group were perfused 4 weeks after injection to examine the fate of the labeled cells; at this survival time, labeled cells have extended axons (Stanfield and Trice, 1988; Hastings and Gould, 1999), survived a period of cell death (Gould et al., 1999b), and begun to express mature neuronal markers (Cameron et al., 1993). All brains were processed for BrdU immunohistochemistry.

**Experiment 2: High doses of BrdU.** Higher doses of BrdU were given to look for a plateau in cell number not found in the first experiment. Young adult rats (300 g, 9–10 weeks old; male rats reach adulthood, as defined by a plateau in the growth rate, at postnatal day 40 according to Wray and Hoffman, 1986) each received a single injection of BrdU (Boehringer-Mannheim, 10 mg/ml in 0.007N

NaOH/0.9% NaCl) at a dose of 300 ( $n = 9$ ), 450 ( $n = 6$ ), or 600 mg/kg ( $n = 9$ ). Groups of rats ( $n = 3$ ) in each dose group were perfused either 24 hours or 4 weeks after injection, as in the first experiment. In addition, three rats in the 300 and 600 mg/kg dose groups were perfused 2 hours after injection; this survival time allows cells to incorporate BrdU, but is not long enough for cells to die and be removed (Thomaidou et al., 1997). Whole brains from the 24-hour survival group and half-brains from the 4-week survival group were processed for BrdU immunohistochemistry. Remaining half brains from the 4-week survival group were processed for thin section multiple antibody immunohistochemistry.

### Experiment 3: Short survival after BrdU injection.

A very short survival time after BrdU injection was used to determine whether dose differences in labeling in the previous experiments were due to prolonged incorporation of BrdU at higher doses. That is, a higher dose of BrdU might be available for uptake longer than a lower dose and might, therefore, label significant numbers of cells not in S-phase at the time of the injection. Young adult rats (300 g, 9–10 weeks old) each received a single injection of BrdU (Boehringer-Mannheim, 10 mg/ml in 0.007N NaOH/0.9% NaCl) at a dose of 50, 100, 300, or 600 mg/kg ( $n = 3$  for all groups). Rats in each dose group were perfused 30 minutes after injection. All brains were processed for BrdU immunohistochemistry.

**Experiment 4: Cell cycle time calculation.** The cell cycle time of dividing cells in the dentate gyrus of young adult rats (300 g, 9–10 weeks old) was calculated by using a modification of the method of Hayes and Nowakowski (2000). For double labeling, five rats received an i.p. injection of [ $^3$ H]thymidine (7 mCi/kg, NEN Life Sciences, 70–90 Ci/mmol), followed 4 hours later by an injection of BrdU (300 mg/kg, Boehringer-Mannheim, 10 mg/ml in 0.007N NaOH/0.9% NaCl), and were perfused 30 minutes after the BrdU injection. For cumulative labeling, five additional rats were injected with BrdU (300 mg/kg, Boehringer-Mannheim, 10 mg/ml in 0.007 N NaOH/0.9% NaCl) five times at 6-hour intervals (injection at  $t = 0, 6, 12, 18,$  and  $24$  hours,  $t_0 = 12$  PM) and perfused 30 minutes after the last injection. Half-brains of all rats were processed for BrdU immunohistochemistry. Remaining half-brains of [ $^3$ H]thymidine-injected rats were processed for thin section combined BrdU-immunohistochemistry and [ $^3$ H]thymidine autoradiography. A third group of six rats was injected with 300 mg/kg BrdU twice per day, every other day, for 8 days (injection at  $t = 0.0, 0.5, 2.0, 2.5, 4.0, 4.5, 6.0,$  and  $6.5$  days,  $t_0 = 8$  AM). Injections were given twice each day to label all dividing cells in the dentate gyrus and were given on alternate days to decrease potential toxicity to other organs. Three of these rats were perfused 5 days after the final injection, and the remaining rats were perfused 12 days after the final injection. All brains were processed for BrdU and neuronal marker immunohistochemistry.

### Tissue processing

Brains were dissected from the skulls and post-fixed in 4% paraformaldehyde (pH 7.4). For all experiments involving BrdU immunohistochemistry alone, coronal sections (50  $\mu$ m) though the entire dentate gyrus of whole or half brains were cut on a sliding microtome. Series of every 12th section through each brain were processed for BrdU immunohistochemistry with DAB according to a

previously published protocol (Cameron and McKay, 1999); in this protocol, sections are mounted on slides, heated in citric acid (0.1 M, pH 6.0) for antigen retrieval, and treated with trypsin and HCl before following standard immunohistochemical staining with monoclonal mouse anti-BrdU antibody (1:100, Becton-Dickinson) and the ABC kit (Vector). Immunohistochemistry was performed simultaneously on one series from each rat to maximize the reliability of comparisons across doses and survival times. All sections were counterstained with cresyl violet and cover-slipped under Permount.

For multiple antibody immunohistochemistry of the 4-week survival brains for BrdU and neuronal markers in experiment 2, half-brains were embedded in Paraplast and cut in 10  $\mu$ m coronal sections on a rotary microtome. Sections were deparaffinized and processed for fluorescent immunohistochemistry according to a previously published protocol (Cameron and McKay, 1999) using the same pretreatment as above, monoclonal rat anti-BrdU (Accurate, 1:100) and either monoclonal mouse anti-NeuN (Chemicon, 1:50) or polyclonal rabbit anti-NSE (Polaron, 1:200), and Cy2-conjugated (1:200) and Cy3-conjugated (1:500) secondary antibodies (Jackson ImmunoResearch, all adsorbed against several species for multiple labeling). Sections were cleared and cover-slipped under Permount. The brains injected several times in experiment 4 were cut in 50- $\mu$ m coronal sections on a rotary microtome. Floating sections were pretreated only with 2 N HCl and stained with either monoclonal rat anti-BrdU (Accurate, 1:200) and mouse anti- $\beta$ -tubulin (TuJ1, BabCo, 1:500) or mouse anti-BrdU (Becton-Dickinson, 1:200) and rabbit anti-TUC-4 (ab25, gift from S. Hockfield, 1:10,000), followed by Alexa 488-conjugated and Alexa 568-conjugated secondary antibodies (Molecular Probes, 1:500) against the appropriate species. Sections were counterstained with bisbenzimidazole and cover-slipped under 70% glycerol.

For combined BrdU immunohistochemistry and [ $^3$ H]thymidine autoradiography in experiment 3, the left half of each brain was embedded in paraffin, and 4- $\mu$ m coronal sections were cut through the caudal half of the dentate gyrus. Sections were deparaffinized and processed for DAB BrdU immunohistochemistry as described above for BrdU immunohistochemistry alone. Sections were then dipped in NTB-2 emulsion, exposed for 20 days in the dark at 4°C, developed in D-19, rinsed, fixed in Kodak Fixer, rinsed, counterstained in cresyl violet, dehydrated, cleared in HemoDe (Fisher), and cover-slipped under Permount.

### Data analysis

In all sections processed for BrdU immunohistochemistry alone, BrdU-labeled cells were counted on coded slides using stereologic methods. BrdU-labeled cells were counted at 400 $\times$  (Olympus BH-2) in the granule cell layer (defined as any cell touching other cells in the layer) and hilus on every 12th section through the entire dentate gyrus, according to a modified version of the fractionator method (Guillery and Herrup, 1997; West, 1993). The choroid plexus and median eminence were examined but not counted, as controls for BrdU injection and staining. Darkly labeled cells were clearly visible in both the choroid plexus and median eminence of nearly all sections that contained these structures. However, two brains in experiment 1 (one 25 mg/kg and

one 100 mg/kg) had no BrdU-labeled cells in any sections; no further analysis was done on these brains. Staining and cell counting was repeated in three series (approximately 25 half-sections per brain) for the 24-hour survival group and two series (approximately 16 whole-sections per brain) for the 4-week survival group in experiment 1, one series for experiment 2 (approximately 8 half-sections per brain), three series (approximately 24 whole-sections per brain) for experiment 3, and two series (16 half-sections per brain) for the cumulatively labeled brains in experiment 4. For each series, the total number of BrdU-labeled cells (bilateral) was estimated by multiplying the number of cells counted by 12 (whole brain sections) or 24 (half-brain sections); the values for multiple repetitions were averaged. Data were subjected to one-way analysis of variance followed by Fisher's least significant difference post hoc comparisons.

In sections labeled with two antibodies, BrdU-labeled cells were counted and categorized as immunoreactive or nonimmunoreactive for the neuronal marker. Cells were additionally categorized according to location in the granule cell layer (touching other cells in the layer), subgranular zone (within two cell body diameters from the edge of the granule cell layer), or hilus (further than two cell body diameters from the granule cell layer). For NSE analysis, 124 cells were counted; 195 cells were analyzed for NeuN, 804 cells for TUC-4, and 324 cells for TuJ1. Percentages of BrdU-labeled cells that were also labeled with neuronal markers were calculated for each brain.

In sections processed for autoradiography, cells labeled with [ $^3$ H]thymidine only, BrdU only, or both markers were counted in the dentate gyrus of 12 anatomically matched sections from each brain. Cells with five or more silver grains were counted as labeled; this value was greater than 50 times background level. Totals and ratios of each cell type were calculated for each brain.

Brightfield images were collected using a SPOT camera (Diagnostic Instruments) or MicroLumina camera (Leaf-systems), and fluorescent images were collected by using a Zeiss 510 confocal microscope. Photo figures were assembled in Adobe Photoshop 5.0.

## RESULTS

### Experiment 1: Low doses of BrdU

Two previously used doses of BrdU (50 and 100 mg/kg) were compared with lower (25 mg/kg) and higher (300 mg/kg) doses to determine the relationship between BrdU dose and cell labeling in the adult dentate gyrus. Differences in the appearance of BrdU-immunoreactive cells were observed across dose groups. Cells in 300 mg/kg group were very dark (Fig. 1), whereas cells in the 25 mg/kg group were very lightly stained and, in many instances, difficult to see clearly. Cells in the 50 and 100 mg/kg groups had intermediate staining (Fig. 1). Although the intensity of staining of cells within a section often varied, all cells were clearly labeled or unlabeled at doses higher than 25 mg/kg. The staining intensity was similar after 24-hour and 4-week survival.

In all dose groups, some BrdU-labeled cells had uniformly stained nuclei, whereas others had a punctate, or spotted, appearance reflecting heterochromatin stain-

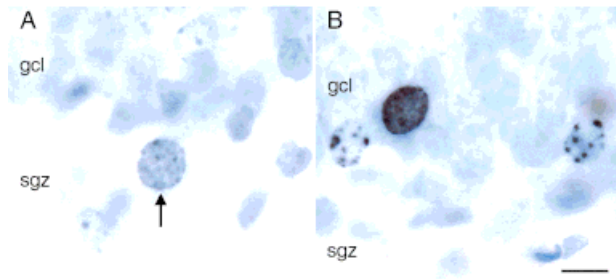


Fig. 1. The appearance of bromodeoxyuridine (BrdU)-labeled cells at different BrdU doses. **A:** Light gray BrdU-labeled cell (arrow) in the adult dentate gyrus of a rat injected with 50 mg/kg of BrdU. **B:** Dark gray BrdU-labeled cell and two cells with a punctate BrdU-staining pattern in a rat injected with 300 mg/kg of BrdU. Blue-purple counterstain in both frames is cresyl violet. gcl, granule cell layer; sgz, subgranular zone. Scale bar = 10  $\mu$ m in B (applies to A,B).

TABLE 1. Effects of BrdU Dose on the Appearance and Survival of BrdU-Labeled Cells in the Dentate Gyrus<sup>1</sup>

| BrdU Dose | % Punctate      |                 | # BrdU+ 4 wk/24 hr |
|-----------|-----------------|-----------------|--------------------|
|           | 24 hours        | 4 weeks         |                    |
| 25 mg/kg  | 25.0 $\pm$ 25.0 | 13.7 $\pm$ 5.7* | 0.22 $\pm$ 0.11*   |
| 50 mg/kg  | 26.2 $\pm$ 3.5  | 29.4 $\pm$ 1.5  | 0.54 $\pm$ 0.16    |
| 100 mg/kg | 23.6 $\pm$ 3.2  | 37.7 $\pm$ 1.3  | 0.68 $\pm$ 0.09    |
| 300 mg/kg | 23.1 $\pm$ 2.9  | 59.3 $\pm$ 0.5* | 0.54 $\pm$ 0.07    |

<sup>1</sup>Values indicate mean  $\pm$  SEM, each from three animals. The data were subjected to one-way analysis of variance with Fisher's least significant difference post hoc tests. \*Significant difference ( $P < 0.05$ ) from all other groups.

ing (Fig. 1). As with solidly stained cells, punctate staining appeared very dark in high dose groups and lighter with lower doses of BrdU. There was no difference in the frequency of punctate staining between dose groups at the 24-hour time point; approximately 25% of the cells in all groups had this appearance (Table 1). This indicates that the punctate pattern is not related to the amount of BrdU incorporated into a cell. Instead, the small constant proportion of cells with this appearance is consistent with the conclusion of Takahashi et al. (1992), based on staining patterns in different subregions of the embryonic ventricular zone and earlier evidence that heterochromatin is replicated at the end of S-phase (Lima-de-Faria and Jaworska, 1968), that punctate staining occurs in cells that are in the latter part of S-phase when BrdU is injected. After a 4-week survival period, 60% of the cells in the 300 and 600 mg/kg groups had punctate staining (Table 2), probably reflecting the clumping of a large proportion of the DNA into heterochromatin in certain mature cell types, including granule neurons. Only 15% of BrdU-labeled cells in the 25 mg/kg group had this appearance (Table 1). The reason for the dose-dependency of punctate staining at the 4-week time point is not clear.

Surprisingly, the number of detectable BrdU-labeled cells increased across the entire dose range at the 24-hour time point (Fig. 2), suggesting that commonly used doses of BrdU do not visibly label all S-phase cells. Statistically significant differences were found between all dose groups, with the exception of 50 mg/kg vs. 100 mg/kg, and the number of labeled cells at 300 mg/kg was more than twice the value obtained with 50 mg/kg. The numbers of BrdU-labeled cells also increased across

TABLE 2. Effects of High Doses of BrdU on the Survival and Neuronal Marker Expression of BrdU-Labeled Cells in the Dentate Gyrus<sup>1</sup>

| BrdU dose (mg/kg) | BrdU-labeled cells |                  |                 | Double-labeled at 4 weeks |                  |
|-------------------|--------------------|------------------|-----------------|---------------------------|------------------|
|                   | 2 hours            | 24 hours         | 4 weeks         | NeuN                      | NSE              |
| 300               | 4,912 $\pm$ 671    | 8,616 $\pm$ 639  | 4,616 $\pm$ 721 | 74.0 $\pm$ 6.0%           | 55.4 $\pm$ 12.3% |
| 450               | —                  | 8,920 $\pm$ 1219 | 5,040 $\pm$ 881 | 83.2 $\pm$ 1.5%           | 53.5 $\pm$ 4.4%  |
| 600               | 4,680 $\pm$ 673    | 9,720 $\pm$ 696  | 4,992 $\pm$ 254 | 74.5 $\pm$ 8.8%           | 77.1 $\pm$ 10.8% |

<sup>1</sup>Values indicate mean  $\pm$  SEM, each from three animals. The data were subjected to one-way analysis of variance. No significant differences were observed between dose groups for any measure. BrdU, bromodeoxyuridine; NSE, neuron-specific enolase.

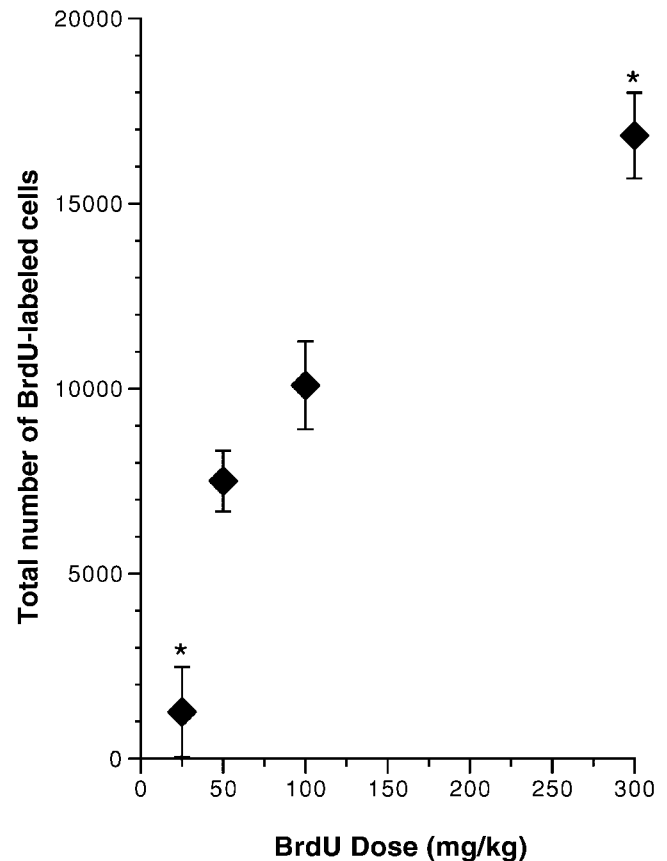


Fig. 2. The number of labeled cells depends on bromodeoxyuridine (BrdU) dose. The graph shows the total number of BrdU-labeled cells in the dentate gyrus (bilateral) after a single injection of BrdU 24 hours earlier. Asterisks indicate significant difference ( $P < 0.05$ ) from all other groups.

doses at the 4-week survival time. To control for differences in initial labeling (observed at 24 hours), the number of BrdU-labeled cells in each animal at 4 weeks was divided by the mean cell count for that dose at 24 hours (Table 1). A significantly lower proportion of cells remained after 4 weeks in the 25 mg/kg group than in the higher dose groups, probably reflecting BrdU dilution below the limits of detection in more cells at this low dose. However, the absence of a decrease in this ratio with higher BrdU dose indicates that the long-term survival of these cells is not negatively affected by BrdU dose across this range.

### Experiment 2: High doses of BrdU

Higher doses of BrdU (300, 450, and 600 mg/kg) were given to determine the dose at which labeled cell number reaches a plateau and to further investigate BrdU toxicity. BrdU-labeled cells in all dose groups were very intensely stained. Fewer labeled cells were found at the 300 mg/kg dose in this experiment compared with experiment 1 (Table 2; Fig. 2). This difference most likely reflects the older age of the animals in the current experiment, as proliferation in the dentate gyrus decreases across the lifespan of the rat (Seki and Arai, 1995).

No significant differences in the numbers of BrdU-labeled cells were observed across dose groups (Table 2) at either the 2-hour or 24-hour survival time, indicating that BrdU labeling plateaus at or below 300 mg/kg. This plateau suggests that doses equal to or higher than 300 mg/kg label all S-phase cells.

There were twice as many BrdU-labeled cells after 24-hour survival as after 2-hour survival with both 300 mg/kg and 600 mg/kg doses; the ratio of the 24-hour value to the 2-hour value was  $1.84 \pm 0.14$  at 300 mg/kg and  $1.98 \pm 0.14$  at 600 mg/kg. Nearly all of the BrdU-labeled cells in the 24-hour group appeared in closely apposed pairs, suggesting daughter cells, and no BrdU-labeled pyknotic cells were observed, providing no evidence for BrdU-induced cell death between 2 hours and 24 hours, like that seen in the embryonic mouse brain (Bannigan and Langman, 1979; Bannigan, 1985). The finding that the number of labeled cells doubles during this 22-hour period also suggests that the entire population of BrdU-labeled cells is dividing, arguing against the possibility that some of the BrdU labeling occurs as a result of DNA repair rather than DNA synthesis. No differences in BrdU-labeled cell number were observed across doses after 4-week survival, either, indicating an absence of long-term BrdU toxicity as well at these doses. The finding that the proportion of BrdU-labeled cells remaining after 4 weeks,  $56 \pm 4\%$  averaged over doses from 50 to 600 mg/kg, is very similar to the 60% value observed in a previous study using [<sup>3</sup>H]thymidine (Cameron et al., 1993) also supports this conclusion. The lack of BrdU toxicity does not mean that all BrdU-labeled cells survive for 4 weeks; the 4-week value is known to reflect a significant amount of death of newly born cells (Gould et al., 1999b). It is impossible to determine the number or proportion of newly born cells that die, because additional cycles of cell division (Cameron et al., 1993), and probably dilution of BrdU below the level of detection, also occur during this interval. These results do indicate, however, that BrdU does not cause or facilitate death of the newly born cells in the adult dentate gyrus.

Because previous studies have found that BrdU can interfere in neural cell differentiation *in vitro* (Prasad et al., 1973; Morris, 1973; Younkin and Silberberg, 1976), we looked for evidence that BrdU altered normal differentiation and/or maturation of new neurons in the adult brain. When sections from the 4-week survival time were double labeled for BrdU and the neuronal markers NeuN or neuron-specific enolase (NSE), the percentage of double-labeled cells in the granule cell layer was not significantly different across dose groups (Table 2). Overall,  $77 \pm 3\%$  of BrdU-labeled cells were NeuN labeled and  $62 \pm 6\%$  were NSE labeled. These percentages are within the range of those previously reported, 41–82% for NeuN and 50–75%

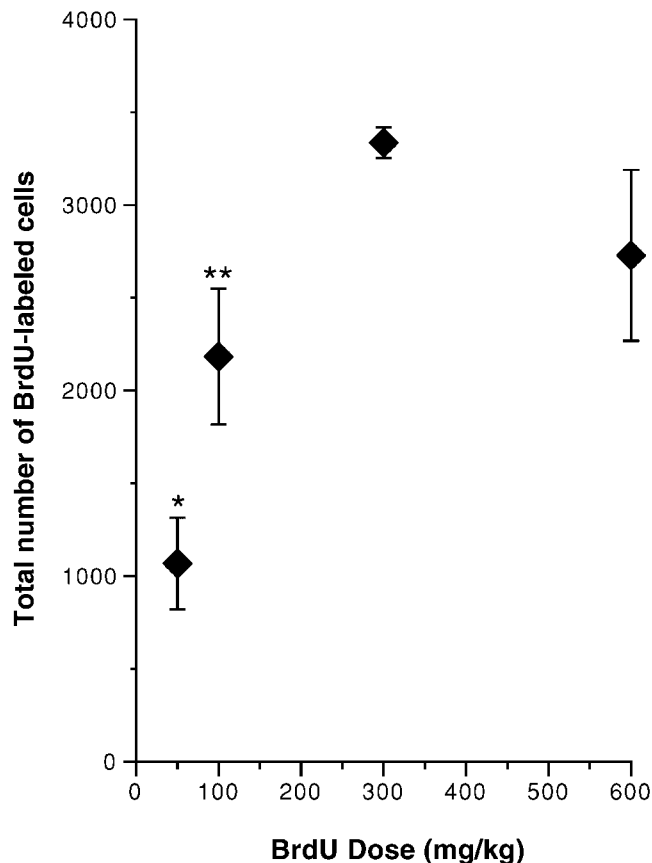


Fig. 3. A short pulse of bromodeoxyuridine (BrdU) labels in a dose-dependent manner. The graph shows the total number of BrdU-labeled cells in the dentate gyrus (bilateral) after a single injection of BrdU 30 minutes earlier. The single asterisk indicates significant difference from all other groups ( $P < 0.05$ ). The double asterisks indicate significant difference from 300 mg/kg group ( $P < 0.05$ ).

for NSE, by using [<sup>3</sup>H]thymidine or lower doses of BrdU (Cameron et al., 1993, 1995; Kempermann et al., 1998; van Praag et al., 1999). The current data, therefore, provide no evidence for an effect of BrdU on differentiation or maturation of neurons in the adult dentate gyrus.

### Experiment 3: Short survival time after BrdU injection

It could be argued that in the previous two experiments, higher BrdU doses labeled more cells due to prolonged availability of BrdU over some threshold dose and subsequent labeling of cells not in S-phase at the time of injection. If this were the case, one would not expect to see an effect of BrdU dose with a short survival time, such as 30 minutes. In this experiment, however, we found that the numbers of BrdU-labeled cells observed after a 30-minute survival period were significantly different across dose groups (Fig. 3), indicating that the higher BrdU doses are indeed labeling more of the cells that are in S-phase at the time of BrdU injection. As in experiments 1 and 2, the 300 and 600 mg/kg groups did not differ, whereas 50 and 100 mg/kg doses resulted in significantly fewer BrdU-labeled cells than the high doses. These results suggest that maximal BrdU labeling of S-phase cells in the adult dentate

gyrus requires a dose higher than 100 mg/kg. A dose of 300 mg/kg is sufficient to label all of the cells, but the actual start of the plateau is likely to occur at some point between 100 and 300 mg/kg.

Fewer BrdU-labeled cells were observed in this experiment than at comparable doses in experiments 1 and 2; these differences are most likely due to the older age of these rats (compared with those in experiment 1) and the shorter survival time in this experiment, which does not allow for the completion of mitosis and may also not be enough time for the entire dose of BrdU to be incorporated.

#### Experiment 4: Cell cycle time calculation

The large numbers of S-phase cells observed with single injections of high dose BrdU suggested that many new granule cells are generated in the adult dentate gyrus. To determine how many cells are being generated, it is necessary to know both the total number of dividing cells in the region and their cell cycle time. Both of these parameters were estimated in young adult rats (9 weeks old, 300 g) by using double labeling with BrdU and [<sup>3</sup>H]thymidine to determine the length of their S-phase and cumulative BrdU labeling to identify the total number of dividing precursors as well as the proportion of cells in S-phase at a given time (Fig. 4).

In rats injected first with [<sup>3</sup>H]thymidine and 4 hours later with BrdU, cells labeled with either marker alone and with both markers were observed (Fig. 5). The number of cells labeled with [<sup>3</sup>H]thymidine alone,  $N_t$ , in 12 sections through each brain was  $24.6 \pm 4.4$ ; these are the cells that were in S-phase at the time of the first injection but left S-phase before the second injection. The total number labeled with BrdU alone,  $N_b$ , in the same sections was  $13.2 \pm 2.2$ ; these cells entered S-phase between the first and second injection. Finally, the number labeled with both BrdU and [<sup>3</sup>H]thymidine,  $N_{bt}$ , was  $45.0 \pm 4.7$ ; these cells were in S-phase at the time of the first injection and remained in S-phase during the 4-hour interval. The ratio of cells labeled with the first label alone ( $N_t$ ) to all cells labeled with the second label ( $N_b + N_{bt}$ ),  $0.433 \pm 0.077$ , is equal to  $i/T_S$  (Fig. 4a), where  $T_S$  is the length of S-phase and  $i$  is the interinjection interval (Hayes and Nowakowski, 2000). Since the interinjection interval was 4 hours, the length of the S-phase for this population is 9.5 hours.

No BrdU-labeled cells appeared to be in M phase; however, a small number of [<sup>3</sup>H]thymidine-labeled mitotic figures were observed (Fig. 5). Because no BrdU-labeled mitotic figures were observed at post-BrdU-injection survival times of 30 minutes, 1 hour, or 2 hours in experiments 1–3, or at 1- and 2-hour time points after [<sup>3</sup>H]thymidine or BrdU injection in several earlier studies (Cameron et al., 1993, 1995, 1998; Cameron and Gould, 1994; Cameron and McKay, 1999), the appearance of small numbers of [<sup>3</sup>H]thymidine-labeled mitotic figures at 4.5 hours suggests that this is a good estimate for the length of G2+M phases,  $T_{G2+M}$ , in this population. Although 4.5 hours is somewhat longer than the approximately 2-hour  $T_{G2+M}$  in many dividing central nervous system populations (Cai et al., 1997), it is consistent with a previous study, which found a range of 4–5 hours for  $T_{G2+M}$  in the early postnatal dentate gyrus (Lewis, 1978). This timing is important for the calculation of  $T_S$  above, because if  $T_{G2+M}$  was shorter than the post-

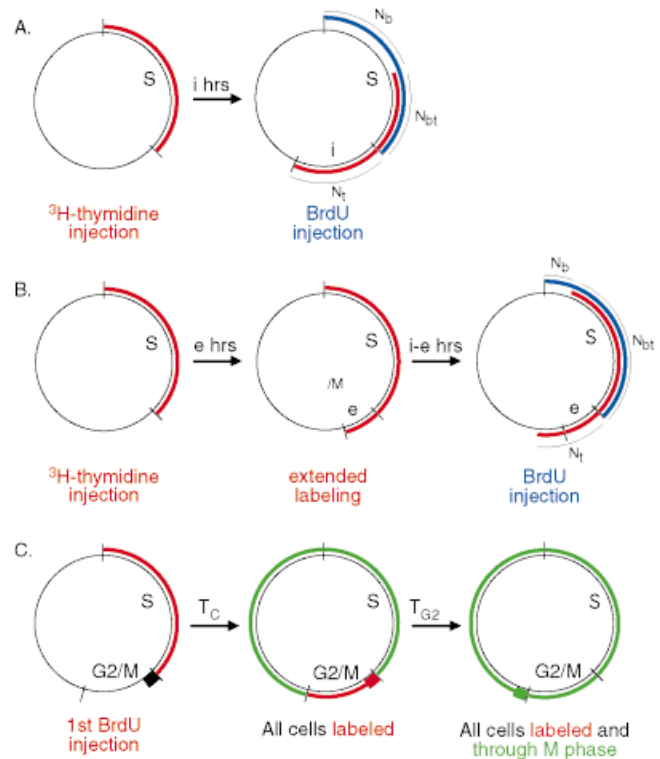


Fig. 4. Design of experiment to determine the length of S-phase and the availability time of [<sup>3</sup>H]thymidine. **A:** A single injection of [<sup>3</sup>H]thymidine labels all cells in S-phase. Four hours later, some [<sup>3</sup>H]thymidine-labeled cells have moved out of S-phase, and some new cells have entered S-phase. Bromodeoxyuridine (BrdU) will label all cells in S-phase at the second time point. The ratio of cells labeled only with [<sup>3</sup>H]thymidine ( $N_t$ ) to all cells labeled with BrdU ( $N_b + N_{bt}$ ) equals the ratio of the interinjection interval,  $i$ , to the time of S-phase. **B:** Because [<sup>3</sup>H]thymidine does not act as a true pulse, new cells coming into S-phase continue to be labeled for a time,  $e$ . If the interinjection interval is longer than  $e$ , new S-phase cells will stop being labeled with [<sup>3</sup>H]thymidine, so some cells will label only with BrdU ( $N_b$ ). The ratio of the extended labeling time,  $e$ , to the time of S-phase ( $T_S$ ) equals the ratio of extra cells labeled with [<sup>3</sup>H]thymidine ( $N_t - N_b$ ) to all cells labeled with BrdU ( $N_b + N_{bt}$ ). **C:** When BrdU is injected multiple times with intervals shorter than  $T_S$ , all cells will become labeled. The last cells to be labeled, shown as a thick line, will be labeled and complete mitosis at a time equal to the length of the cell cycle plus the length of the G2/M phases,  $T_C + T_{G2/M}$ . With cumulative labeling times shorter than this, not all cells will complete mitosis after being labeled, whereas with times longer than this, some cells will complete mitosis twice.

[<sup>3</sup>H]thymidine-injection survival interval, some labeled cells would have completed mitosis, increasing  $N_t$  and leading to an underestimation of  $T_S$ . This was not likely to be a problem in this experiment, because the survival interval was essentially equivalent to  $T_{G2+M}$  but not longer.

It has long been believed that [<sup>3</sup>H]thymidine and BrdU are cleared from the body, and presumably the brain, very rapidly, i.e., within 30 minutes (Packard et al., 1973); consequently an injection of one of these markers would act as a "pulse," labeling a discrete cohort of S-phase cells. However, a recent embryonic study suggests that, in fact, both markers may continue to label cells in the brain for a considerably longer time, 5–6 hours (Hayes and Nowa-

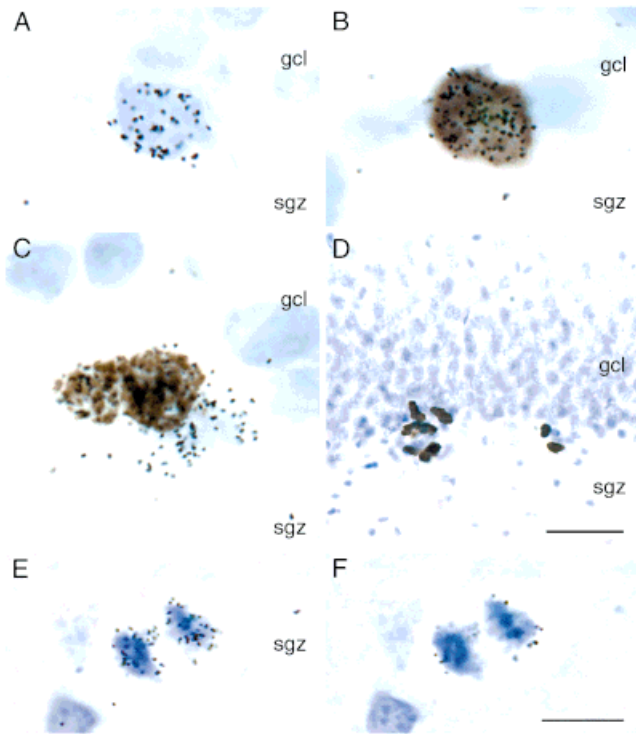


Fig. 5. Cells in the adult dentate gyrus labeled with [ $^3\text{H}$ ]thymidine, bromodeoxyuridine (BrdU), or both. **A:** A cell labeled only with [ $^3\text{H}$ ]thymidine left S-phase between the [ $^3\text{H}$ ]thymidine injection (4.5 hours before perfusion) and the BrdU injection (0.5 hours before perfusion). **B:** A cell labeled with both [ $^3\text{H}$ ]thymidine and BrdU remained in S-phase between the two injections. **C:** A cluster of cells with different labeling indicates that cell clusters do not divide synchronously. **D:** BrdU-labeled cells in cumulatively labeled brains (five BrdU injections at 6-hour intervals) are found in clusters like this one containing 17 cells (several not in the plane of focus) or in pairs. **E,F:** A [ $^3\text{H}$ ]thymidine-labeled mitotic figure, with silver grains (E) and anaphase chromatin (F) in different focal planes indicates that 4.5 hours is enough time for cell labeled in S-phase to reach M phase. Blue-purple counterstain in all frames is cresyl violet. Scale bars = 40  $\mu\text{m}$  in D; 10  $\mu\text{m}$  in F (applies to A–C,E,F).

kowski, 2000). In the current study, we were able to calculate the labeling time of [ $^3\text{H}$ ]thymidine in the adult dentate gyrus in the following manner. If an injection of [ $^3\text{H}$ ]thymidine acted as a true pulse, labeling only those cells in S-phase at the moment of injection, the number of cells leaving S-phase during the interinjection interval should equal the number entering S-phase during this same interval:  $N_t = N_b$ . However, the data show that fewer cells were labeled with [ $^3\text{H}$ ]thymidine alone than with BrdU alone, indicating that [ $^3\text{H}$ ]thymidine continues to label cells during some portion of the interinjection interval. The proportion of “extra” thymidine-labeled cells should be equivalent to the ratio of the extended labeling time ( $T_e$ ) to S-phase time ( $T_S$ ), i.e.,  $(N_t - N_b)/(N_b + N_{bt}) = T_e/T_S$  (Fig. 4b). By using the values above for  $N_t$ ,  $N_b$ ,  $N_{bt}$ , and  $T_S$ ,  $T_e$  equals 1.9 hours, indicating that [ $^3\text{H}$ ]thymidine continues to label S-phase cells in the adult dentate gyrus for almost 2 hours. This extended labeling time explains previous findings that the number of [ $^3\text{H}$ ]thymidine-labeled cells in the adult dentate gyrus doubles between 2 and 24 hours after injection (Okano et al., 1996) but more

than doubles between 1 hour and 24 hours (Cameron et al., 1993); the 1.9-hour labeling time means that additional cells are labeled after the 1-hour survival time, but not after 2 hours. Similar evidence from the current study, that the BrdU-labeled cell number at 24 hours is twice the number at 2 hours (experiment 2), indicates that BrdU also stops labeling cells in the adult brain within 2 hours, in agreement with studies showing that BrdU clearance time is identical to that of thymidine (Packard et al., 1973; Hayes and Nowakowski, 2000).

The total number of BrdU-labeled cells counted in serial 50- $\mu\text{m}$  sections through the dentate gyrus of rats given a single injection of BrdU 30 minutes before perfusion,  $N_s$ , was  $3,598 \pm 228$ . In cumulatively labeled brains, labeled at 6-hour intervals for 24 hours and killed 0.5 hours after the final injection, the number of BrdU-labeled cells in the dentate gyrus,  $N_{co}$ , was  $16,937 \pm 1,020$ . Because many of the cells labeled by the early injections will have completed mitosis producing two labeled daughters, this observed number of BrdU-immunoreactive cells is larger than the number of cells that were initially labeled. A total cumulative labeling time equal to  $T_C + T_{G2/M}$  is required for all cells to become labeled and then complete M phase (Fig. 4c). The proportion of cells, then, that will incorporate BrdU and complete mitosis during cumulative labeling is given by  $(\text{labeling time} - T_{G2/M})/T_C$ . For a labeling time of 24.5 hours and  $T_{G2/M}$  of 4.5, this is equivalent to  $20/T_C$ . The remaining portion of the cells,  $1 - 20/T_C$ , will not be doubled. This means that  $N_{co}/N_c = 2(20/T_C) + 1(1 - 20/T_C)$ , or  $N_{co}/N_c = (20 + T_C)/T_C$ . If  $T_C$  is 20 hours, all cells would complete mitosis exactly one time after becoming labeled, and the observed labeled cell number will be twice the number of cells initially labeled in S-phase. A longer  $T_C$  would result in some of the cells not completing M phase and not being doubled, and, conversely, a shorter  $T_C$  would result in some cells completing mitosis twice. The ratio  $N_s/N_{co}$ , which represents the proportion of dividing cells in S-phase at a given time, is equivalent to the proportion of cell cycle time represented by S-phase:  $N_s/N_{co} = T_S/T_C$  (Hayes and Nowakowski, 2000). Combining this equation with the one relating  $N_c$  and  $N_{co}$ , and substituting our previously calculated value of 9.5 hours for  $T_s$ , gives the equation  $T_C = (9.5N_{co} - 20N_s)/N_s$ . Plugging in the observed values for  $N_{co}$  and  $N_s$  from above,  $T_C = 24.7$  hours. The size of the proliferating pool,  $N_c$ , is 9,355 cells. If 9,355 progenitor cells proliferate with a cell-cycle time of 24.7 hours, 9,089 new cells will be produced each day and 276,457 cells will be generated each month.

The cell cycle time calculated above was used to design an injection paradigm for examining the early differentiation, using markers that are expressed early in neuronal maturation, of a cohort of newly born cells labeled with BrdU over the course of 1 week. Rats were injected twice a day; based on a 24.7-hour cell cycle and a 1.9-hour extended labeling time, this should be sufficient to label all dividing cells in the adult dentate gyrus. Injections were given on alternate days to try to decrease potentially harmful effects to other organ systems, although this may have also resulted in loss of some labeled cells due to BrdU dilution. Two different survival periods were used, 5 days and 12 days after the last injection, but because no significant differences were found between the two groups for any measure, data from the two groups were pooled. BrdU-labeled cells were found in all subregions of the

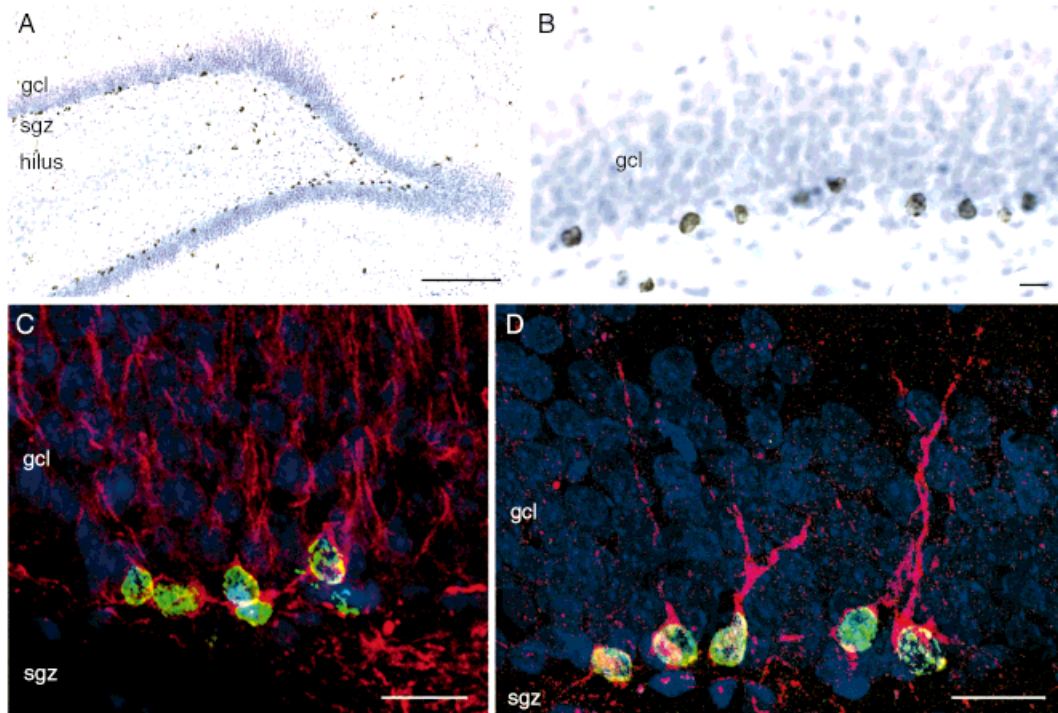


Fig. 6. Large numbers of neurons are born in 1 week. **A:** The dentate gyrus of a rat injected with bromodeoxyuridine (BrdU) multiple times over a 1-week period has a few gray/black BrdU-labeled cells within the hilus and many in the granule cell layer (gcl) and subgranular zone (sgz). **B:** A higher power image shows that most of the BrdU-labeled cells (gray/black) lined up on the edge of the granule cell layer are the size and shape of granule cells. **C:** Confocal z-stack

image (10  $\mu\text{m}$  total) of BrdU-labeled cells (green) in the granule cell layer double-labeled with the neuronal marker TuJ1 (red). **D:** Confocal z-stack image (17.6  $\mu\text{m}$  total) of neurons in the granule cell layer double-labeled with BrdU (green) and the immature neuronal marker TUC-4 (red). A cell with immature morphology can be seen on the far left. Blue-purple counterstain is cresyl violet in A,B and bisbenzamide in C,D. Scale bars = 200  $\mu\text{m}$  in A; 20  $\mu\text{m}$  in B–D.

dentate gyrus, with  $44 \pm 4\%$  of the labeled cells located in the granule cell layer, another  $30 \pm 3\%$  in the subgranular zone, and the remaining  $26 \pm 2\%$  in the hilus. Nearly all of the BrdU-labeled cells in the granule cell layer ( $94 \pm 1\%$ ) expressed the early neuronal marker class III  $\beta$ -tubulin, as detected by the antibody TuJ1 (Fig. 6). Ab25 against TUC-4 (TOAD/ulip/crmp-4, formerly TOAD-64, Quinn et al., 1999), which is expressed specifically by immature neurons (Minturn et al., 1995), double-labeled  $84 \pm 4\%$  of BrdU-labeled cells in the granule cell layer (Fig. 6). Throughout the dentate gyrus as a whole, 51% and 52% of BrdU-labeled cells were labeled by TuJ1 and ab25, respectively. The remaining BrdU-labeled cells probably represent a combination of progenitor cells and microglia and/or oligodendrocytes; few, if any, astrocytes are normally born in the adult dentate gyrus (Cameron et al., 1993). Although cells that were clearly endothelial were not included in the counts, some labeled endothelial cells may not have been recognized and excluded. Dendritic morphology characteristic of dentate gyrus granule cells could be observed in many of the TUC-4-labeled cells; these neurons had a single primary dendrite that branched one or two times, characteristic of granule cells in the deep portion of the granule cell layer (Fig. 6), whereas some others had an immature appearance, with a bipolar or stellate morphology and some dendrites projecting parallel to, rather than through, the granule cell layer (Fig. 6). The neuronal morphology and neuronal marker

expression of large numbers of BrdU-labeled cells along the edge of the granule cell layer indicates that a significant number of new granule cells are produced each week.

## DISCUSSION

### BrdU is a specific, nontoxic marker of S-phase cells in the adult dentate gyrus

There is a potential concern that BrdU, particularly at high doses, could visibly label cells undergoing DNA repair as well as those synthesizing DNA as part of mitosis. However, several observations suggest that cells repairing DNA were not visibly labeled in the current study. First, the number of cells labeled with a single injection of BrdU doubles between 2 hours and 24 hours after injection (experiment 2), indicating that BrdU must be labeling only dividing cells. The only way to gain labeled cells after BrdU is cleared from the brain (within 2 hours) is for the cells to complete mitosis. If additional labeled cells at higher doses reflect DNA repair rather than more S-phase cells, this extra subpopulation of cells would not double and the total number of labeled cells at the 24-hour time point would be less than twice the number at 2 hours. Second, a previous study has shown that the level of DNA repair in the normal brain, as measured by [ $^3\text{H}$ ]thymidine autoradiographic grain counts, is remarkably constant throughout all neurons of the dentate gyrus granule cell



layer and the hippocampus, as well as in other neuronal populations in the brain (Korr and Schultze, 1989; Korr et al., 1989; Schmitz et al., 1999). Therefore, labeling due to DNA repair should be uniformly observed in virtually all neurons in the dentate gyrus. BrdU staining in the current study was not consistent with this pattern: staining was confined to a small subset of cells in the proliferative region of the dentate gyrus (the subgranular zone), and cells in the outer portion of the granule cell layer and in the pyramidal cell layer were never labeled. Although higher doses of BrdU labeled more cells, the location of the labeled cells was not altered.

Although DNA repair has been visualized using BrdU immunohistochemistry (Selden et al., 1993; 1994), there were important methodologic differences between these studies and studies of cell proliferation. Immunohistochemical labeling of DNA repair has only been reported in cultured nonneuronal cells, after DNA damage produced by genotoxic chemicals or exposure to ultraviolet irradiation. This type of damage is repaired through a pathway that replaces approximately 100 nucleotides at each damaged site; normally occurring *in vivo* DNA damage is repaired through a pathway that replaces only 1–2 nucleotides at each site, resulting in much lower levels of BrdU incorporation (see Schmitz et al., 1999 for review). A recent experiment (Palmer et al., 2000) using gamma irradiation, which produces DNA damage similar to that which occurs *in vivo* but at a higher rate (Schmitz et al., 1999), found no detectable BrdU immunohistochemical labeling of cultured fibroblasts, and a review of the literature shows no published reports of BrdU labeling of DNA repair *in vivo*. Another important difference between the two types of studies is that more sensitive methods are used to detect the label in the DNA repair studies. For *in vivo* autoradiographic detection of DNA repair, exposure times are 12 times longer (240 days vs. 20 days) than the exposure generally used for studies of proliferation (Korr and Schultze, 1989; Korr et al., 1989; Schmitz et al., 1999). For the immunohistochemical studies, the exposure to BrdU lasted very long, 24–48 hours compared with the approximately 2-hour period during which BrdU is likely to be available in the brain after systemic injection. These immunohistochemical studies also used a unique anti-BrdU antibody (clone IU-4) that forms stable bonds with individual molecules of incorporated BrdU; the vast majority of anti-BrdU antibodies require divalent antigen binding, which occurs only with a relatively high proportion of local BrdU substitution or 2.5% overall substitution, neither of which is likely through the normal *in vivo* repair mechanism (see Selden et al., 1994 for review). Therefore, although small amounts of BrdU are likely to be incorporated into all cells in the brain due to DNA repair, this low level of incorporation was not detected in the current study, even at the highest doses of BrdU.

The lack of adverse effects of BrdU in this study contrasts with deleterious effects of BrdU on dividing cells in the developing brain observed in several previous studies. Evidence for BrdU-induced cell death in the embryonic brain has been found with maternal doses of 60 mg/kg (two 60 mg/kg injections, 6 hours apart, Kolb et al., 1999) and 400–600 mg/kg, but not 300 mg/kg (Bannigan, 1985; Webster et al., 1973; Bannigan and Langman, 1979). Because the dose that provides optimal labeling of S-phase cells in the brain appears to be six times higher in adults than in embryos, it may be that the toxic dose is also six

times higher. However, it is clear from the current study that studies of adult neurogenesis in the dentate gyrus can be conducted without concern for toxic effects of BrdU on the newly born neurons.

### Blood-brain barrier

The results presented here indicate that a higher systemic dose of BrdU is required to optimally label S-phase cells in the adult brain than in the embryonic brain. The most likely explanation for this result is that the blood-brain barrier, which develops around P10 in rats (Johanson, 1989), limits the entry of BrdU into the brain. This explanation is supported by the appearance, even at the lowest dose of BrdU (25 mg/kg), of darkly stained cells in circum-ventricular organs, such as the median eminence and choroid plexus, which are circumventricular organs lacking a blood-brain barrier.

Previous studies of neurogenesis in adult mammals have found evidence for a blood-brain barrier to thymidine in cats (Das and Altman, 1971) and primates (Kaplan, 1983) by comparing *i.p.* injections to *i.c.v.* injections. The blood-brain barrier is especially tight in the rat compared with several other species (Bradbury, 1979), so thymidine would not be expected to diffuse freely into the brain in this species either. Although it was once believed that thymidine entered the brain through the blood-cerebrospinal fluid barrier at the choroid plexus (Spector and Berlinger, 1982), recent studies show that thymidine enters the brain primarily through facilitative low-affinity, high-capacity carrier-mediated nucleoside transport systems at the blood-brain barrier and that the CSF normally functions as a sink for removal of thymidine (Thomas and Segal, 1996; Thomas et al., 1997). BrdU transport into the brain has not been examined *in vivo*, but *in vitro* studies suggest that BrdU is transported by the same active and facilitative nucleoside transport systems as thymidine (Lynch et al., 1977; Spector, 1982; Spector and Huntoon, 1984).

### High doses of BrdU are required to label all S-phase cells in the adult dentate gyrus

Initially, because of concerns about potential BrdU toxicity, we tested the most commonly used BrdU dose, 50 mg/kg, against higher and lower doses to determine the lowest dose that would be sufficient to label all S-phase cells in the dentate gyrus of mature rats. Surprisingly, the number of visibly labeled cells increased across the entire dose range rather than reaching a plateau. Further experimentation showed a plateau in BrdU-labeled cell number that appeared at 300 mg/kg. Lower doses of BrdU, *i.e.*, 100, 50, and 25 mg/kg, label 60%, 45%, and 8% of S-phase cells, respectively. The plateau in cell number strongly suggests that doses greater than or equal to 300 mg/kg are labeling all S-phase cells. However, it should be noted that it is impossible to directly determine whether all S-phase cells in this region are being visibly labeled, because S-phase cells in this region are scattered among cells in other phases of the cell cycle as well as large numbers of postmitotic neurons and glial cells (Cameron et al., 1993). Therefore, it is possible in principle that the plateau in cell number occurs due to saturation of the nucleoside transporter systems and that even the high doses of BrdU do not label all S-phase cells.

Because all previously published studies of adult neurogenesis with BrdU have used doses less than 300 mg/kg,

the amount of neurogenesis in the adult dentate gyrus appears to have been significantly underestimated in the past. A small number of experiments have been done using a dose of 200 mg/kg (Hastings and Gould, 1999; Gould et al., 1999b); because this dose lies in an untested part of the curve determined in the current study, it is possible that it is also sufficient for maximal labeling. The vast majority of studies, however, have used very low doses, less than 100 mg/kg, and have, therefore, labeled fewer than half of the S-phase cells. This issue is not likely to be a problem for most experiments, which compare two or more treatment groups given the same dose of BrdU. Relationships between treatment groups in numbers of BrdU-labeled cells should be constant, even if only a proportion of the S-phase cells in each group are detected. There is a potential problem, however, in any experiment involving groups in which the concentrations of BrdU entering the brain may be different. For example, comparing rates of proliferation in adult brains to those in developing animals that lack a blood-brain barrier may be difficult. Different species have differences in the permeability of the blood-brain barrier to many compounds (Bradbury, 1979), probably related to differences in the densities of nucleoside transporters (reviewed in Thomas and Segal, 1997), that also make it problematic to compare neurogenesis between species. Additionally, treatments that disrupt the blood-brain barrier, e.g., kainate or seizures (Oztas et al., 1992; Pont et al., 1995), or alter the flow of blood to the brain, e.g., exercise (Ide and Secher, 2000), could produce increases in BrdU-labeled cell number independent of changes in proliferation by altering BrdU availability in the brain. It should be possible to avoid these problems if BrdU doses are high enough that all S-phase cells are labeled in all treatment groups. The current findings suggest that a dose of 300 mg/kg is high enough to avoid this type of problem in the dentate gyrus, as long as none of the treatments decrease entry of BrdU into the brain. It should be noted that this dose is not necessarily sufficient for studies of other brain regions; local differences in the permeability of the blood-brain barrier to nucleosides and population-specific cell cycle kinetics, e.g., a longer S-phase, could mean that higher concentrations of BrdU are required to label all S-phase cells in other parts of the brain.

### **Large numbers of neurons are produced in the adult dentate gyrus**

By determining the length of the cell cycle for dividing cells and the total number of dividing cells, we calculated that approximately 9,000 new cells are generated in the adult rat dentate gyrus each day. This number is 18% of the estimated rate of 50,000 new cells per day generated at the peak of granule cell proliferation (Schlessinger et al., 1975). Throughout the 2- to 3-week developmental peak of proliferation, the cell cycle time of dentate gyrus granule cell progenitors appears to remain fairly constant around 16 hours (Lewis, 1978; Nowakowski et al., 1989). The longer cell cycle time (24.7 hours) in the current study suggests that there is a "developmental" increase in cell cycle time, as seen during neocortical neurogenesis (Takahashi et al., 1995), but that it occurs over an extended period of time, reflecting the extended neurogenetic period of this population. This progressive lengthening of the cell cycle may continue throughout adulthood. The number of dentate gyrus cells labeled with a single injection of BrdU

decreases across the entire life span of the rat (Seki and Arai, 1995); this decrease was also observed in the current study between the 5-week-old rats in experiment 1 and the 10-week old rats in experiments 2 and 3. Because the age-related decrease in BrdU-labeling in rats can be reversed by removing adrenal steroids (Cameron and McKay, 1999), it seems likely to reflect a progressive lengthening of the cell cycle time in the dentate gyrus with age as opposed to shrinking of the size of the progenitor population. Additionally, hourly and daily changes in levels of factors such as stress, corticosteroids, estrogen, glutamatergic excitatory input, serotonin, and opioids all regulate neurogenesis in this region (Cameron and Gould, 1994; Cameron et al., 1995; Gould et al., 1997; Brezun and Daszuta, 1999; Tanapat et al., 1999; Eisch et al., 2000); the rapid time course of this regulation suggests that these factors also produce their effects through alterations in the length of the cell cycle. Therefore, the length of the cell cycle calculated for untreated 3-month-old rats is probably not static, but a constantly changing value that reflects the animal's age, history, and current environment.

Many newly generated cells normally die under standard laboratory conditions between 1 and 2 weeks after they are born (Gould et al., 1999b). Before this time, however, they develop marker expression profiles and dendrites characteristic of granule neurons (current study) and extend axons into CA3, the normal granule cell target area (Hastings and Gould, 1999). Electrophysiological evidence suggests that granule cells with dendrites characteristic of very young, TUC-4-expressing, granule cells exhibit robust LTP (Wang et al., 2000). Taken together, this evidence indicates that the new cells are likely to function as neurons before the period of cell death and suggests that they may be engaging in competitive survival. The survival of newly born granule cells is very sensitive to environmental manipulations; both hippocampal learning tasks (Gould et al., 1999b) and larger cages with more rats and novel objects (Kempermann et al., 1998) dramatically decrease the number of newly born cells that die during this early period. This finding suggests that in naturalistic settings, which are much more complex than laboratory conditions, death of newly born neurons may be minimal. If most of these young cells were to survive 4 weeks, the numbers of young neurons in the dentate gyrus could be as large as 138,000, or 6% of the total granule cell population of 2.4 million (West et al., 1991). However, the size of this immature population relative to the mature granule cell population may not be the most important comparison, because the new cells may not function primarily as part of the mature population, e.g., to increase its size or to replace lost cells. Instead, young neurons in the adult dentate gyrus might act as a distinct functional neuronal population, since evidence suggests that they may have different electrophysiological properties from the mature granule cells, including significantly increased LTP and insensitivity to GABAergic inhibition (Liu et al., 1996; Wang et al., 2000). The number of granule neurons generated in a month is 62% as large as the afferent population of entorhinal cortex stellate cells (Mulders et al., 1997) and 28% as large as the efferent CA3 pyramidal cell population (West et al., 1991). A neuronal population of this size with distinct properties is clearly large enough to play an important role in the function of the dentate gyrus.

## ACKNOWLEDGMENTS

We thank Susan Hockfield for the generous gift of ab25 (TUC-4) antibody, and Richard Sleboda for excellent technical assistance. This study was funded by the Intramural Research Program of NINDS, NIH.

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