

DEVELOPMENT AND DISEASE

Early determination and long-term persistence of adult-generated new neurons in the hippocampus of mice

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Accepted 11 October 2002

SUMMARY

New neurons are continually generated in the adult hippocampus, but the important question, whether adult neurogenesis is transient or leads to the lasting presence of new neurons, has not yet been answered. Dividing cells were labeled with bromodeoxyuridine (BrdU) and were investigated by means of immunofluorescence and confocal microscopy at several time-points 1 day to 11 months thereafter. BrdU-labeled neurons remained stable in number and in their relative position in the granule cell layer over at least 11 months. This finding implies that the addition of new neurons is not transient and that their final number and localization are determined early. By contrast,

expression of immature markers β -III-tubulin and doublecortin in BrdU-labeled cells, peaked early after division and was not detectable after 4 weeks. In transgenic mice expressing enhanced green fluorescent protein under the nestin promoter none of the BrdU/nestin-positive cells early after division expressed the mature marker NeuN, confirming that no dividing neurons were detected. These new data suggest that new neurons are recruited early from the pool of proliferating progenitor cells and lead to a lasting effect of adult neurogenesis.

Key words: Stem cell, Progenitor cell, Adult neurogenesis, Mouse

INTRODUCTION

In adult hippocampal neurogenesis new neurons are generated throughout life and added to the granule cell layer (GCL) of the dentate gyrus (Altman and Das, 1965; Cameron and McKay, 1999; Cameron et al., 1993; Kaplan and Hinds, 1977; Kuhn et al., 1996). Growing evidence supports a role for adult hippocampal neurogenesis in hippocampal function in health and disease. Our theory is that adult neurogenesis allows the neuronal network of the dentate gyrus to be optimized according to functional demands (Kempermann, 2002b). In addition, there is support for adult hippocampal neurogenic dysfunction in major depression or dementia (Duman et al., 2000; Jacobs et al., 2000; Kempermann, 2002a). In this context, the as yet unanswered question of whether the new neurons are persistent or transient becomes particularly important.

Neurogenesis is defined as the series of developmental steps that leads from the division of a neural stem or progenitor cell to a mature, functionally integrated neuron. Therefore, quantification of 'neurogenesis' and its regulation is influenced by the time, at which it is measured during this development.

Some studies have only assessed cell proliferation as a

measure of neurogenesis; however, cell proliferation in the subgranular zone (SGZ) also leads to gliogenesis and angiogenesis (Palmer et al., 2000) and most of the new cells die (Biebl et al., 2000; Kempermann and Gage, 2002b; Kempermann et al., 1997a). Investigation of several time-points after cell division is required to obtain a reasonable estimate of neurogenesis. Most studies on adult hippocampal neurogenesis have followed the new cells for about 4 weeks after division. This survival time is sufficient for the cells to become recognizable as neurons or glial cells by immunohistochemistry. Van Praag et al. (Van Praag et al., 2002) showed that the electrophysiological properties of the new granule cells are indistinguishable from the older cells, but also found evidence that full maturation might take additional weeks or even months.

In addition, the answer to the question of whether the new neurons are generated transiently or persistently influences functional interpretations of adult neurogenesis. There is good, although still correlational evidence that functional stimuli promote the survival of new hippocampal neurons (Gould et al., 1999; Kempermann et al., 1997b; Nilsson et al., 1999). We theorize that the functional role of adult hippocampal

neurogenesis lies in enabling the hippocampus to cope better with novelty and to adjust the dentate gyrus to processing new and greater levels of complexity (Kempermann, 2002b). Adult neurogenesis cumulatively and strategically adds to the neuronal network in the dentate gyrus, in order to optimize its functionality at the smallest possible size (Kempermann, 2002b). This hypothesis is supported by the fact that the genetically determined baseline levels of adult hippocampal neurogenesis correlate with the acquisition of the water-maze task, but not with the recall of the newly learned information (Kempermann and Gage, 2002a). Several studies indicate that the total number of granule cells increases over the first year of life in a rodent (Bayer, 1985; Boss et al., 1985). Consequently, our morphological hypothesis is that adult hippocampal neurogenesis leads to a fast, stable and lasting integration of new neurons.

To test this hypothesis, we labeled dividing cells with the thymidine analog bromodeoxyuridine (BrdU) and studied the numbers of BrdU-labeled cells in the dentate gyrus at various time-points after division in an attempt to assess a time-course of cellular survival, migration and differentiation. We analyzed the distribution of phenotypes among the newly generated cells in order to understand better at what ratio transiently expressed neuroectodermal markers give way to markers associated with cellular maturation.

MATERIALS AND METHODS

Animals and housing conditions

Thirty-five female C57BL/6 mice, ~2 months old at the beginning of the experiment, were obtained from Harlan Sprague Dawley. A second set of mice, $n=10$, ~2 months old, consisted of transgenic mice expressing enhanced green fluorescent protein under the nestin promoter (Yamaguchi et al., 2000). All mice were kept according to standard governmental and NIH regulations, five per cage. They had access to water and food ad libitum and lived in a 12 hour dark/light cycle. (Fig. 1 and Table 1 provide an overview of the two sets of mice and their treatment.)

BrdU injections

For 12 consecutive days, all mice in the first set received one daily intraperitoneal injection of 10 mg/ml BrdU (5-bromo-2-deoxyuridine; Sigma) in sterile 0.9% NaCl solution (daily dose: 50 μ g/g body weight). From the first set of mice, 5 animals each were perfused 1 day, 3 days, 7 days, 4 weeks, 3 months, 6 months and 11 months after the last injection of BrdU, as described below. The transgenic animals in set 2 received two injections of BrdU (50 μ g/g body weight) 6 hours apart and were perfused 2 hours or 24 hours later.

Tissue preparation

The mice were killed with an overdose of ketamine and perfused transcardially with 4% paraformaldehyde in cold 0.1 M phosphate buffer. The brains were stored in the fixative overnight and then transferred into 30% sucrose. One day later, 40 μ m coronal sections were cut from a dry ice-cooled block on a sliding microtome (Leica). The sections were stored at -20°C in cryoprotectant containing 25% ethylene glycol, 25% glycerin and 0.05 M phosphate buffer.

Antibodies

All antibodies were diluted in TBS containing 0.1% Triton X-100, 0.05% Tween 20 and 3% donkey serum (TBS-plus).

The primary antibodies used in this study were: monoclonal rat anti-BrdU (Harlan Seralab), 1:500; monoclonal mouse anti-NeuN

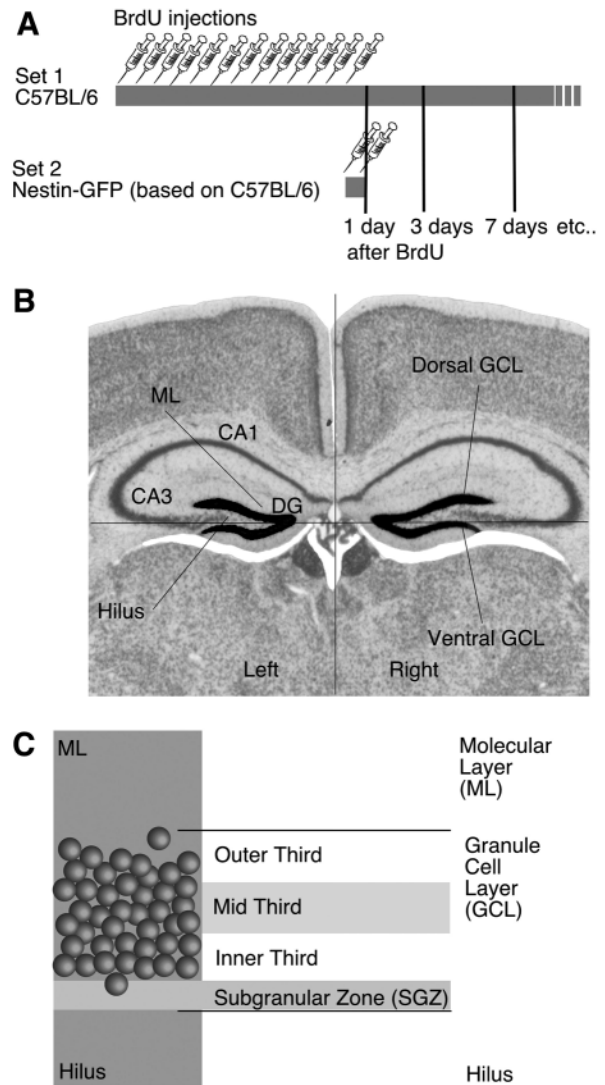


Fig. 1. Experimental design. (A) Labeling of newly generated cells was achieved by injecting proliferation marker BrdU into the mice. Cumulative labeling with 12 injections over 12 days leads to a large number of BrdU-positive cells, but results in decreased temporal resolution. A valid estimate of the size of the proliferating population is not possible. (Set 2) To estimate the amount of proliferation at a specific time-point, two single injections of BrdU (6 hours apart) were given to transgenic mice expressing green fluorescent protein (GFP) under the nestin promoter (Yamaguchi et al., 2000). The mice were perfused 24 hours later (see also Table 1). (B) Numbers of BrdU-marked cells were categorized according to their localization in the two blades (dorsal and ventral) of the dentate gyrus in both hemispheres. The underlying image of the mouse brain section is taken from the Mouse Brain Library, section 19 (Rosen et al., 2000), available at <http://mickey.utmem.edu/MBL/mb1.html>. (C) Numbers of BrdU-marked cells within the GCL were also classified depending on whether they were found in the SGZ plus the inner third of the GCL, or the mid third or the outer third of the GCL. The SGZ was defined as a two-nucleus-wide band below the apparent border between the GCL proper and the hilus.

(Chemicon), 1:100; monoclonal mouse anti-doublecortin (Santa Cruz); monoclonal mouse anti- β -III-tubulin (Promega) and polyclonal rabbit-anti S100 β (SWant, Bellinzona, Switzerland), 1:2000; and polyclonal rabbit-anti GFP (Abcam), 1:400.

Table 1. Experimental groups

Set	Strain	Age	BrdU injections	Examination after	Characteristics assessed
1	C57	2 months	×12	1, 3 and 7 days; 4 weeks; 3, 6 and 11 months	Survival, migration and differentiation
2	Nestin-GFP	2 months	Two (6 hours apart)	1 day	Proliferation

Two sets of mice were examined in this study. Set 1 ($n=35$) received 1 daily of injection of BrdU for 12 consecutive days and five mice were killed and examined at each of the seven different time-points listed. The GFP-expressing transgenic mice of Set 2 received only two injections of BrdU, 6 hours apart, and were perfused 24 hours later.

For indirect immunofluorescence the following secondary antibodies were used (all 1:250): donkey anti-rabbit IgG (Jackson) conjugated with CY5 or FITC; donkey anti-mouse IgG (Jackson) conjugated with FITC or CY5; and goat anti-rat IgG (Jackson) conjugated with Rhodamine-X. For immunohistochemistry with the peroxidase technique, biotinylated donkey anti-mouse IgG (Jackson) (1:250) was used as secondary antibody and detected with avidin-biotin-peroxidase complex (ABC, Vectastain Elite, Vector Laboratories) (9 μ l/ml).

Pretreatment for BrdU immunohistochemistry

After quenching endogenous tissue peroxidases with 0.6% H_2O_2 in TBS for 30 minutes the sections were incubated in 2 N HCl for 30 min at 37°C and washed in 0.1 M borate buffer (pH 8.5) for 10 minutes.

Immunohistochemistry

For light microscopic quantification of BrdU-labeled cells a series of every sixth 40 μ m section was used. After BrdU pretreatment (see above) and washing in TBS, sections were blocked in TBS-plus with 3% horse serum for 1 hour, followed by incubation in primary antibody in TBS-plus overnight at 4°C. After rinses in TBS, the sections were incubated in the secondary antibody in TBS-plus for 4 hours at room temperature. After another set of rinses, ABC Elite reagent (Vector Laboratories) was applied for 1 hour. As substrate for the peroxidase reaction, diaminobenzidine (DAB, Sigma) was applied for 5 minutes at a concentration of 0.25 mg/ml in TBS with 0.01% hydrogen peroxide and 0.04% nickel chloride. Sections were thoroughly washed, mounted, air dried and coverslipped.

Immunofluorescence

For immunofluorescent triple-labeling of BrdU, NeuN and S100 β ; BrdU, β III-tubulin and S100 β ; and of BrdU, doublecortin (DCX) and NeuN every twelfth section throughout the dentate gyrus was used. After pretreatment (see above) and a blocking step with TBS-plus containing 3% donkey serum, sections were incubated in a mixture of the three antibodies of each series for 36 hours at 4°C. After washing in TBS and TBS-plus, a cocktail of secondary antibodies (Rhodamine X to detect BrdU, FITC for NeuN, DCX, and β -III-tubulin, and CY5 for S100 β and NeuN) was applied for 4 hours at room temperature. Sections were washed again, mounted and coverslipped in polyvinyl alcohol with diazabicyclo-octane (DABCO) as an anti-fading agent.

Fluorescent signals were detected using a confocal laser scanning microscope (Leica TCS SP2). For each series the phenotypes of 50 BrdU-labeled cells per animal were determined. Images were processed with Adobe Photoshop 6.0 (Adobe Systems).

Quantification

Sampling of BrdU-positive cells was done exhaustively throughout the GCL in its rostrocaudal extension. Cells were categorized according to their localization in the dentate gyrus of both sides (see Fig. 1B for details; numbers for set 2 animals represent the left hemisphere only). As BrdU-labeled cells are comparatively rare, the stereological procedure, described elsewhere (Kempermann et al., 1997a; Williams and Rakic, 1988), was modified to exclude the

uppermost focal plane only. The resulting number of BrdU-positive cells was then multiplied by 6 (because every sixth section had been used) to give an estimate of the total number of BrdU-positive cells.

Statistical analyses

All statistical analyses were performed with Statview 4.5.1 for Macintosh. Factorial ANOVA was performed for all comparisons of morphological data followed by Fisher post hoc test, where appropriate.

RESULTS

The number of BrdU-labeled cells declines early after division, but then stabilize

The experimental design is outlined in Fig. 1 and Table 1. To study the time-course of the survival of newly generated cells in the adult dentate gyrus the numbers of BrdU-labeled cells were determined at several time-points after they had incorporated BrdU into their DNA during cell division (Fig. 2). The number of BrdU-labeled cells in the dentate gyrus, including the subgranular layer and irrespective of phenotype, was determined at 1 day, 3 days, 1 week, 4 weeks, 3 months, 6 months and 11 months after the last injection of BrdU. Cumulative labeling with proliferation marker BrdU over a period of 12 days resulted in a large number of BrdU-labeled cells 1 day after the last injection of BrdU. The number of BrdU-labeled cells decreased and became more or

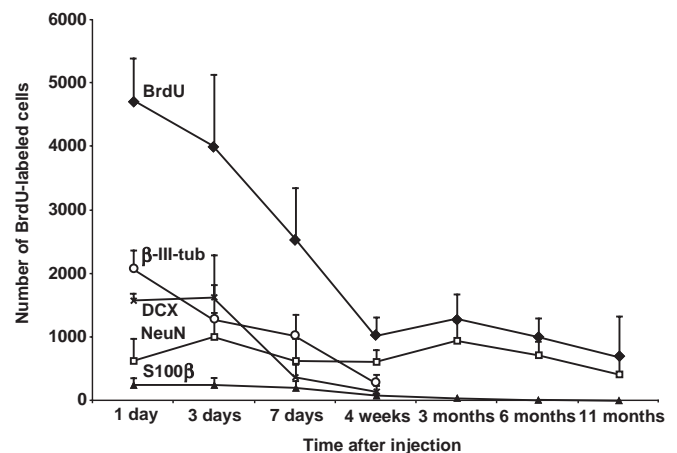


Fig. 2. Number of BrdU-labeled cells at various time-points after BrdU injection. BrdU was given once a day for 12 consecutive days. The time-points of investigation reflect 1 day, 3 days, 7 days, etc., after the last injection of BrdU. Numbers are absolute cell counts per animal. The other curves depict the numbers of BrdU-labeled cells that show a co-localization with doublecortin (DCX), β -III-tubulin, NeuN or S100 β .

Table 2. Distribution of BrdU-labeled cells in the dentate gyrus

Area	Mean±s.e.m.	Mean difference	<i>P</i>
Dorsal blade	209.6±31.5	58.5	<0.0001**
Ventral blade	151.1±24.7		
Left GCL	179.9±27.7	-6.2	0.4118
Right GCL	186.1±28.9		

Comparison of left versus right granule cell layer (GCL) and dorsal blade versus ventral blade. See Fig. 1B. n/s, not significant.

**Indicates a significant difference.

less stable after 4 weeks. All numbers in this graph (Fig. 2) reflect counts per animal, i.e. for both hippocampi. The finding of decreasing BrdU counts after labeling concurs with earlier reports (Biebl et al., 2000; Kempermann et al., 1997a). The decrease is not linear. Although there was a steep decline over the first few days after the last injection of BrdU, the decrease leveled out over time. There was no significant difference between the numbers at 4 weeks and 11 months after injection ($P=0.7107$): cells that were present at 1 month after the last injection of BrdU were likely to be present after 11 months.

Only limited differences in the regional distribution of new cells in the dentate gyrus over time

BrdU-positive cells were categorized as to whether they were found in the left or right hippocampus and whether they were located in the dorsal or the ventral blade of the dentate gyrus (Fig. 1B). Table 2 shows that, with regard to the number of BrdU-labeled cells 1 day after the last injection of BrdU, there was no difference between the left and right brain, but significantly more BrdU-labeled cells were found in the dorsal than the ventral blade of the dentate gyrus. If analyzed at each individual time-point these difference were only significant at some of the time-points (3 months, $P=0.0156$; 6 months, $P=0.0433$) and had low *P* values at two additional time-points (1 day, $P=0.0890$; 4 weeks, $P=0.0622$), suggesting high variability and limited immediate relevance.

Migration of BrdU-labeled cells ends early after division

Migration of BrdU-labeled cells was assessed by categorizing the cells according to their position within the GCL. We

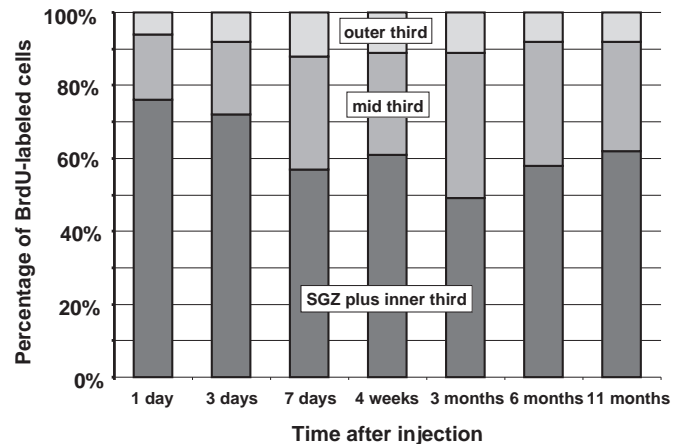


Fig. 3. Distribution of BrdU-labeled cells within the GCL at different time-points after BrdU injection. For details on the spatial categorization, see Fig. 1C.

compared the numbers of labeled cells in (1) the SGZ plus the inner third of the GCL, (2) the mid third of the GCL and (3) the outer third of the GCL (Fig. 1C). The distribution of BrdU-labeled cells in the SGZ and the GCL shows a discrete shift from the SGZ towards the mid third of the GCL during the first 4 weeks. Significant differences between the percentage of cells in the SGZ/inner third of the GCL and the mid and outer third were found with decreasing frequency at 1, 3 and 7 days after BrdU injection (Fig. 3, Table 3). This finding implies that after the first few weeks the surviving cells do not substantially change their position in the GCL.

Not all cells expressing immature neuronal markers become fully mature neurons, but the final number of new neurons is determined early

Young immature neurons express markers such as β -III-tubulin (Geisert and Frankfurter, 1989; Menezes and Luskin, 1994) or doublecortin (DCX) (Cooper-Kuhn and Kuhn, 2002; des Portes et al., 1998; Gleeson et al., 1998). As Figs 2, 4 illustrate, BrdU-labeled cells can show a co-localization with these markers soon after the last injection of BrdU. These numbers reach $46.5\pm 5.6\%$ and 2083 ± 286 in absolute numbers (β -III-tubulin) and $47.5\pm 6.9\%$ and 1629 ± 672 (DCX), but decline thereafter (all numbers are means±s.e.m.). At 3 or 6 months

Table 3. Migration pattern of new neurons

	1 day	3 days	7 days	4 weeks	3 months	6 months	11 months
% of BrdU-labeled cells in SGZ	76.1±2.3	73.0±2.9	57.3±6.9	61.6±5.2	49.6±5.3	58.7±4.6	62.0±7.4
<i>P</i> values for comparisons							
1 day		n/s	0.0170	0.0601	0.0013	0.0264	0.0679
3 days			0.0432	n/s	0.0038	0.0645	n/s
7 days				n/s	0.0432	n/s	n/s
4 weeks					n/s	n/s	n/s
3 months						n/s	n/s
6 months							n/s

A statistical analysis comparing the percentage of BrdU-labeled cells in the subgranular zone plus inner third with the mid plus outer third of the granule cell layer reveals a slow redistribution away from the subgranular zone (see also Fig. 1C and Fig. 3). The upper half of the table gives the percentage of the BrdU-labeled cells in SGZ plus inner third of the GCL (Fig. 3). In the lower half, the *P*-values of the comparisons are listed. There is no indication of a continued migration towards the molecular layer. ANOVA ($F=3.057$): $P=0.0199$. Fisher post-hoc tests; significant *P* values are bold.

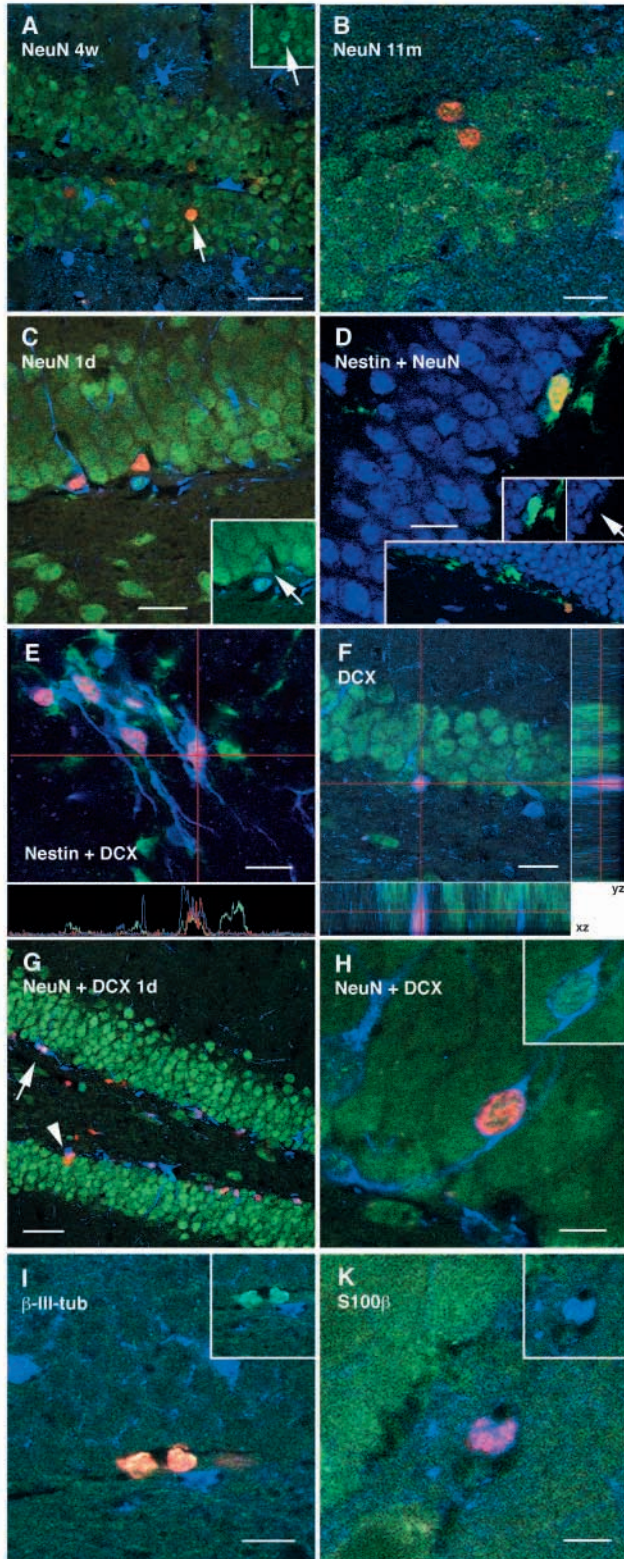


Fig. 4. Phenotypic analysis of BrdU-labeled cells. (A) Four weeks after the last injection of BrdU, cells double-labeled for BrdU and NeuN could be detected. This image confirms previous findings that numerous BrdU/NeuN-positive cells, which are morphologically indistinguishable from the surrounding granule cells can be found 4 weeks after BrdU injection. The inset highlights the double-labeling by showing the immunoreaction for NeuN (arrow) in the BrdU-labeled cell. BrdU/NeuN-positive cells can be found in the entire GCL, not only in the SGZ, indicating early expression of a mature neuronal marker and migration soon after division. NeuN, green; BrdU, red; astrocytic marker S100 β , blue. Scale bar: 80 μ m. (B) Eleven months after BrdU injection, BrdU/NeuN-labeled cells could be found. They were morphologically the same as they were 4 weeks after BrdU injection (compare with B). Generally, the staining intensities are lower in the older animals. The whitish granules in and between the cells are age pigment lipofuscin. NeuN, green; BrdU, red; astrocytic marker S100 β , blue. Scale bar: 20 μ m. (C) BrdU-labeled cells can be detected as early as 1 day after the last (of 12) injection of BrdU (see inset for display of NeuN-immunoreaction, arrow). NeuN, green; BrdU, red; doublecortin (DCX, see below), blue. Scale bar: 25 μ m. (D) Twenty-four hours after a single injection of BrdU, no BrdU/NeuN-labeled cells can be detected (arrow in upper inset; compare with top left inset). Furthermore, NeuN cannot be detected in cells that express intermediate filament nestin as a marker of stem or progenitor cells. Nestin was detected in these mice by expressing green fluorescent protein (GFP) under the nestin promoter (Yamaguchi et al., 2000). The nestin-GFP-expressing putative stem or progenitor cells form clusters along the SGZ (see lower inset). GFP, green; BrdU, red; NeuN, blue. Scale bar: 15 μ m. (E) DCX is a protein expressed in young, maturing neurons. Soon after division, many BrdU-labeled nestin-GFP expressing cells become DCX positive. The lower panel shows the fluorescence intensities along the horizontal line drawn through one of the nuclei. BrdU, red; nestin-GFP, green; DCX, blue. The marked cell is triple-labeled, but the fluorescence intensity is lower than in the right neighboring cell that expresses nestin-GFP only. Scale bar: 25 μ m. (F) Colocalization of BrdU and DCX can be further visualized in z -series through the section (z -distance is 12 μ m). The reconstructed views along the yz -axis (right panel) and xz -axis (bottom panel) demonstrate that red BrdU immunoreaction is surrounded by blue DCX immunoreaction. NeuN, green. Scale bar: 20 μ m. (G) The overview shows that along the SGZ BrdU-positive cells (red) can be found that express DCX (blue) and appear pink (arrow, see also F). Similar to C, some BrdU/NeuN-positive cells (orange, arrowhead) can be seen. DCX-labeled cells are restricted to the SGZ. NeuN, green; BrdU, red; DCX, blue. Scale bar: 80 μ m. (H) Many DCX-expressing cells are NeuN-positive, indicating a temporal overlap between the two markers. DCX-expressing cells often have long processes, extending into the molecular layer. The inset indicates how cytoplasmic DCX expression (blue) engulfs nuclear and perinuclear NeuN-expression (green). BrdU, red. Scale bar: 10 μ m. (I) β -III-tubulin can serve as another marker for immature neurons. At early time-points after BrdU, clusters of BrdU/ β -III-tubulin marked cells can be found. However, β -III-tubulin yields a weaker staining, does not extend into the neurites and cannot be combined with NeuN. β -III-tubulin, green; BrdU, red; S100 β , blue. Scale bar: 20 μ m. (K) New astrocytes can be identified by colocalizing immunoreactivity for BrdU (red) and S100 β (blue). NeuN, green. Scale bar: 15 μ m. Insets in I and K show controls.

after BrdU injection, only occasional cells co-labeled for BrdU and either of these markers could be found. As these double-labeled cells were so rare, they were not explicitly quantified and no data points representing them were included in Fig. 2.

NeuN was used as a marker for mature neurons (Mullen et al., 1992). Interestingly, BrdU/NeuN labeled cells could

already be found at 1 day after the last injection of BrdU. Subsequently, the relative number of new neurons (BrdU+/NeuN+) remained stable over the period investigated (Figs 2, 4). In other words, the total number of BrdU/NeuN double-positive cells did not decrease either with the total number of BrdU-labeled cells or with those showing a

colocalization with immature neuronal markers. By contrast, BrdU-labeled astrocytes (BrdU+/S100 β +) decreased and were almost absent at the 11 months time point.

Although the 12-day injection period increases the number of labeled cells that could be studied, it also reduced the temporal resolution at the early time-points after BrdU injection. The time-point '1 day' after the last injection of BrdU actually reflects a range of 1 to 12 days after BrdU injection. To study an early time point after BrdU injection with higher precision, a second set of mice that allows the immediate recognition of cells with progenitor cell properties was used.

Nestin is an intermediate filament that is expressed in neural stem or progenitor cells and thus can to some degree serve as a progenitor cell marker (Lendahl et al., 1990; Reynolds et al., 1992). In transgenic mice expressing green fluorescent protein (GFP) under the nestin promoter, nestin-expressing cells and thus presumable stem or progenitor cells can be visualized efficiently (Sawamoto et al., 2001; Yamaguchi et al., 2000). These mice received only two injections of BrdU and were perfused 2 and 24 hours later. As detection of GFP is strongly reduced by the treatment with HCl, which is necessary for BrdU immunohistochemistry, we used an antibody against GFP to identify nestin-expressing cells. We found that with this method, 24 hours after BrdU injection, 55% of the BrdU-positive cells were nestin-GFP positive (Fig. 4). Relying on direct GFP emission or using antibodies against nestin led to many fewer double- or triple-labeled cells (not shown). Importantly, at 24 hours after BrdU injection none of the BrdU-positive cells were NeuN positive (Fig. 4).

Two hours after two injections of BrdU, $7.2 \pm 1.7\%$ of the BrdU-labeled nestin-GFP expressing cells were also DCX positive (Fig. 4), further suggesting that cell fate decisions towards neuronal development are made extremely soon after division. At 24 hours after BrdU injection, the number of BrdU/DCX/nestin-GFP triple-labeled cells had increased to $24.8 \pm 6.4\%$, indicating that DCX expression increases over time. By contrast, it is difficult to assess at what time-point nestin itself is cleared from the maturing cells, because it is not known how long the reporter gene product remains detectable after the reporter gene promoter is turned off. However, as illustrated in Fig. 4, the immunohistochemical signal for nestin-GFP expression was consistently lower in BrdU-positive cells expressing DCX than in nestin-GFP positive cells that were positive for BrdU only. This finding might be indicative of either the clearance of nestin itself or at least of nestin-GFP from the cells that are in the process of acquiring a neuronal phenotype.

We also investigated whether DCX-immunoreactive cells would show double labeling for nestin-GFP. We found that of 250 DCX-positive cells counted separately in five animals $28.4 \pm 2.9\%$ displayed nestin-GFP co-staining whereas only $1.2 \pm 1.2\%$ were positive for NeuN. Importantly, NeuN never co-localized with nestin-GFP.

DISCUSSION

In adult hippocampal neurogenesis, the fate of the new neurons is determined early, probably within the first few days after

division. Once this determination is made, the presence of the new neurons remains rather stable. As this statement is based on the appearance of DCX in the new cells, the time-point of decision might occur as early as within the first hours after division (or even during the cell cycle itself), but not all of the cells that express early neuronal markers fully mature into NeuN-positive granule cells.

Our previous research has demonstrated that functional stimuli, such as exposure to a challenging complex environment, increase the number of new neurons by means of a survival-promoting effect (Kempermann et al., 1997b; Kempermann et al., 1998). Presumably this effect would occur very soon after division. We hypothesize that a surplus of immature neuronal cells provides a pool of neurogenic potential from which appropriate functional stimuli, such as environmental complexity or learning stimuli, can recruit more new neurons, resulting in the net increase in neurogenesis seen in these other studies (Gould et al., 1999; Kempermann et al., 1997b; Kempermann et al., 1998). This sensitive 'neurogenic window' might range from a stage at which cells still express nestin only and are undergoing division to a later stage where immature neuronal markers have been expressed.

Our present knowledge about the exact sequence of neuronal development in adult neurogenesis remains limited. Several steps, such as cell proliferation, survival, expression of neuronal markers, neuritogenesis, synaptogenesis, etc., can be conceptually identified. However, no longitudinal examination is possible in an *in vivo* study. All examinations are snapshots in a continuum of largely unknown kinetics. Still, our present data do suggest that the fate choice decision for new neurons is made earlier than previously assumed and that this decision has lasting consequences.

BrdU is a proliferation marker that, in contrast to techniques based on the immunohistochemical identification of cell cycle-related proteins, captures the time point when the compound is circulating in the blood stream, not conditions at the time-point when the animal is killed. This 'birth-dating' property allows us to identify new postmitotic cells that had divided at the known time of BrdU injection. Because NeuN is confirmed as a truly postmitotic marker here (no BrdU+/NeuN+ cells were found 24 hours after 2 injections of BrdU), we can conclude that for those cells that are recruited to become long-lasting neurons the step to a postmitotic state is made soon after division.

The first time-point investigated here, 1 day after the last injection of BrdU, can be considered as a gross estimate of 'proliferative activity' in the SGZ. However, this attribution has several limitations (Hayes and Nowakowski, 2002; Nowakowski et al., 1989). With regard to the size of the proliferating cell population, the cumulative application of BrdU leads to an overestimate. One reason for this lies in the kinetics of BrdU incorporation in relation to cell cycle parameters. Another reason is that spreading out BrdU injections over several days results in a reduction in temporal resolution. Numbers at 'day 1' reflect not only cell proliferation but also to some degree cellular survival. They are also influenced by effects such as dilution of the label due to continued divisions. With the exception of the data from the nestin-GFP transgenic animals that received only two pulses of BrdU within 6 hours and were killed 2 hours and 24 hours later (Set 2; Fig. 1A), all BrdU counts in the present study refer to

cells that have exited the cell cycle within the period of 12 days during which BrdU was injected.

The detection of NeuN in BrdU-positive cells at early time-points after BrdU injection does not imply that mature neurons would divide, because if the neuronal phenotype is determined at 24 hours after BrdU injection, no BrdU/NeuN double-labeled cells were found. This observation is in accordance with data from a study by Palmer et al. (Palmer et al., 2000).

The important issue as to whether BrdU incorporation also reflects DNA repair (at the time when BrdU is available in the blood stream) has been discussed in greater detail elsewhere (Cooper-Kuhn and Kuhn, 2002; Palmer et al., 2000). Briefly, the two main arguments against the possibility that numbers of BrdU-labeled cells such as in our study are heavily confounded by DNA repair are: (1) irradiation that induces DNA strand breaks does not lead to an increased uptake of BrdU in vivo (Parent et al., 1999) and in vitro (Palmer et al., 2000); and (2) adult neurogenesis can also be detected using a retroviral labeling, which would not detect DNA repair (Van Praag et al., 2002). Our present study adds another circumstantial argument, because the data suggest a progression through different markers of neuronal development among the BrdU-labeled cells that is inconsistent with the detection of DNA repair in already matured cells.

Many of the newly generated cells die within the first few days after division (Biebl et al., 2000). Also, the increase in adult neurogenesis induced by exposure to an enriched environment found its counterpart in a reduced level of apoptosis (Kempermann et al., 1997b; Young et al., 1999). These findings fit with data from embryology, wherein great numbers of neurons are produced in surplus and those that are not actively selected for function are eliminated by apoptosis (Blaschke et al., 1996). Most, if not all selective processes that are involved in integrating the new cells into the hippocampal circuits or in eliminating cells that are not used, apparently take place within the first weeks after division. We hypothesize that during adult hippocampal neurogenesis, like during embryogenesis, new but immature neurons are generated in surplus and are then selected into functional circuits. This hypothesis is supported by the fact that larger numbers of DCX- and β -III-tubulin-expressing cells are generated than are those showing NeuN as a more mature marker. Once the consolidation process is over, during which time immature markers remain expressed, the cells become persistently part of the GCL. The net effect is the stable integration of a comparatively low number of neurons. This might imply that adult neurogenesis contributes to a lasting alteration of the hippocampal network, although this addition is small. Our data indicate that investigating 'neurogenesis' at 4 weeks after the application of the proliferation marker allows a valid estimate of long-term effects. However, our data also show that it is not sufficient to use immature neuronal markers such as β -III-tubulin or DCX as indicators of net neurogenesis.

Nestin-GFP-expressing cells begin to express DCX very early after division and the number of cells that express DCX increases thereafter. The switch from the immature marker DCX to the more mature marker NeuN appears to be swift and, at least in a certain percentage of cells, must occur early. However, the large number of BrdU/DCX-labeled cells that can be found during the first few weeks after division suggests that the time-window during which such a switch can occur is

actually much longer. A more detailed future analysis will address this issue.

To date the question of whether or how new neurons in the adult hippocampus migrate has not been extensively studied. It can easily be demonstrated, however, that some migration occurs (Kempermann and Gage, 2002b; Kuhn et al., 1996). In the olfactory system, newly generated cells migrate over a long distance (several millimeters) through the rostral migratory stream to the olfactory bulb (Lois et al., 1996; Luskin, 1993). We show that new neurons in the hippocampus reach their final position very early. The maximum distance the new cells migrate lies within a range of 50 to 100 μ m. However, many of them do not seem to migrate at all and remain in the SGZ.

For embryonic development a classical concept of an outside-in gradient exists with the oldest cells at the molecular layer and the younger cells near the hilus (Altman and Bayer, 1990; Crespo et al., 1986). A study by Martin et al., used chimeras to study the clonal composition of the dentate gyrus and found that the later-born cells clearly preferred the inner core of the GCL (Martin et al., 2002). Our data relate to this finding in that we demonstrate that, in the adult brain, the majority of new cells remains confined to the inner third of the GCL. During adult granule cell development a sequential activation of transcription factors can be observed, from Mash1 in putative progenitor cells to NeuroD in young granule cells (Pleasure et al., 2000). In this study, stronger NeuroD expression could be found in the inner half of the GCL, further strengthening the view that later born granule cells preferentially gather in this region. This study is also relevant to the current study because it raises the issue of whether the developmental steps we have seen with regarding to the sequential expression of proteins (nestin to doublecortin to NeuN) could be related to the corresponding expression of transcription factors. Therefore, it will be important to characterize the developmental steps in adult hippocampal neurogenesis with regard to the underlying molecular events.

From the data obtained by Martin et al. (Martin et al., 2002) it can be concluded that different progenitor cell populations form the outer and the inner shell of the GCL. Presumably, the progenitor cells from which adult hippocampal neurogenesis originates constitute yet another population of precursor cells that, as one might expect, relate to the later progenitors of the inner core rather than to the earlier progenitors of the outer shell. According to Altman and Bayer (Altman and Bayer, 1990) the stem or progenitor cells of the SGZ form a tertiary germinative center (after the primary matrix at the ventricle wall and the secondary matrix that forms the outer shell of the GCL).

This organization does not lead to a restricted distribution of new granule cells, because in our study a considerable number of new neurons could be found throughout the entire thickness of the adult GCL. Wojtowicz and co-workers described different electrophysiological properties (as well as changed morphological features) in cells at different distances from the SGZ (Wang et al., 2000). Our data suggest that new neurons migrate very early to a location within the dentate gyrus and stay there. We propose therefore that the position within the GCL is meaningful for the function of the new neurons, and further that the functional differences within the GCL are not a function of cellular age (with increasing age equaling a greater distance from the SGZ) but of location per se.

Accordingly, new neurons find their functionally appropriate place, depending on a concrete functional demand.

Our hypothesis is that new neurons are strategically inserted into the neuronal network of the dentate gyrus, thus enabling the dentate gyrus to process novel and more complex information (Kempermann, 2002b). From this perspective, a fast and lasting integration of the new cells makes sense. But even a functional integration of new neurons within a few days will most likely come too late for processing the information that triggered the neurogenic response. Confrontation with a challenging new situation rather serves as a trigger to adapt the network of the processor to challenges to come. In this sense, both young and old animals benefit from the long lasting integration of new neurons to the dentate gyrus that we have found in the current study. However, it is equally conceivable that old animals that already have had more experience (and thus more opportunities to optimize their hippocampal network for the tasks encountered) can live with reduced levels of adult hippocampal neurogenesis.

VolkswagenStiftung, Deutsche Forschungsgemeinschaft (DFG), National Institute of Aging, Lookout fund and Alexander von Humboldt Foundation (Max Planck Award to F.H.G.) funded this study. We thank Mary Lynn Gage for editing the manuscript and Katja Reuter for valuable support.

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