THE JOURNAL OF COMPARATIVE NEUROLOGY 413:146–154 (1999)

Rapid Extension of Axons Into the CA3 Region by Adult-Generated Granule Cells

NICHOLAS B. HASTINGS* AND ELIZABETH GOULD
Department of Psychology, Princeton University, Princeton, New Jersey 08544

ABSTRACT

The dentate gyrus continues to produce granule neurons throughout adulthood. The present study examined the extension of axons by adult-generated granule neurons into hippocampal area CA3. We injected the fluorescent retrograde tracers Fast blue (FB) and FluoroRuby (FR) into area CA3 of adult male rats at various times after the administration of 5′-bromo-2′-deoxyuridine (BrdU), a marker of proliferating cells and their progeny. We report that immature granule cells extend axons into CA3 as rapidly as 4–10 days after mitosis. A significant increase in the percentage of BrdU-labeled cells that were labeled with FB or FR was observed by 2 weeks after BrdU administration. This proportion remained roughly constant up to 3 weeks after BrdU-labeling, a time at which markers of a mature neuronal phenotype are expressed. BrdU-labeled cells that contained either FB or FR often were located far from the tracer injection site, indicating that these cells had extended relatively long axons. Collectively these results suggest that adult-generated granule neurons may influence normal hippocampal function, even at a very early stage after their production. J. Comp. Neurol. 413:146–154, 1999.

Indexing terms: adult neurogenesis; dentate gyrus; tract tracing; FluoroRuby; Fast blue; bromodeoxyuridine

During adulthood, precursor cells located in the hilus and subgranular zone (sgz) of the dentate gyrus divide and produce daughter cells, the majority of which appear to express morphologic and biochemical characteristics of neurons. This process has been described in a wide range of mammalian species (Cameron et al., 1993; Gould et al., 1997, 1998, 1999b; Kempermann et al., 1997), including humans (Eriksson et al., 1998). However, although the evolutionary conservation of adult neurogenesis suggests that it is biologically significant, its precise role in normal hippocampal function remains enigmatic. For adult-generated neurons to be relevant functionally, they must be incorporated into existing neurocircuitry. Presently, however, very little is known regarding the extent to which this occurs.

Since the earliest demonstrations that cells in the dentate gyrus of adult rodents incorporate tritiated thymidine (^{3}H-thymidine), a marker of cell proliferation (Angevine 1965; Altman and Das, 1967), considerable efforts have been made to identify adult-generated cells positively as neurons. These studies have shown that adult-generated hippocampal cells eventually reside in the granule cell layer (gcl; Cameron et al., 1993; Gould et al., 1997), receive synaptic input (Kaplan and Hinds, 1977; Kaplan and Bell, 1984), assume the nuclear and cytoplasmic morphology of neighboring neurons (Cameron et al., 1993; Gould et al., 1999a), and express biochemical markers of immature (Seki and Arai, 1995; Parent et al., 1997; Scott et al., 1998; Gould et al., 1999b) and, ultimately, mature neurons (Cameron et al., 1993; Gould and Tanapat, 1997; Kempermann et al., 1997).

The elaboration of an axonal process, although it is not strictly a prerequisite, provides convincing evidence of neuronal fate. In an effort to establish the identity of cells generated in the adult rat dentate gyrus, Stanfield and Trice (1988) combined ^{3}H-thymidine autoradiography with retrograde tracing. Those authors observed only a few thymidine-labeled cells that were labeled with retrograde tracer when it had been injected into hippocampal area CA3. Thus, although their report demonstrates that some adult-generated cells extend axons into a target region typical of mature dentate granule neurons, the low number of new tracer-labeled cells observed diminishes the possible functional significance of these adult-generated neurons. In addition, that study examined only new cells 28 days after the administration of ^{3}H-thymidine, a time now known to follow the expression of biochemical markers of mature granule neurons (Cameron et al., 1993;

Grant sponsor: National Institutes of Health; Grant number: MH52423.

*Correspondence to: Nicholas B. Hastings, Department of Psychology, Green Hall, Washington Road, Princeton, NJ 08544.
E-mail: hastings@princeton.edu

Received 16 March 1999; Revised 6 June 1999; Accepted 17 June 1999

Gould and Tanapat, 1997). A more recent retrograde tracing study using the thymidine analog bromodeoxyuridine (BrdU) to label new cells has confirmed the extension of axons into the CA3 region by cells that were generated approximately 12 weeks before perfusion (Markakis and Gage, 1999). Because both of these studies examined single, relatively late time points after BrdU injection, it remains unclear whether axons are characteristic only of mature, adult-generated granule neurons or whether projections also may be formed by new cells at earlier time points.

We sought to determine the point at which the axons of adult-generated granule neurons could first be detected in area CA3 and the proportion of adult-generated hippocampal cells that contribute projections to this region. We therefore injected fluorescent retrograde tracers into area CA3 at time periods after the administration of BrdU that were known to coincide with the expression of morphologic and biochemical characteristics of immature or mature granule neurons.

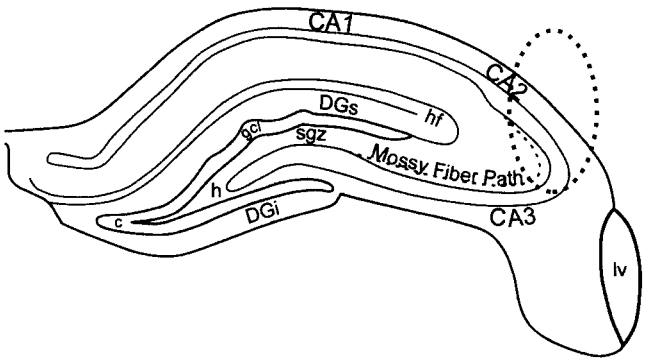

Fig. 1. Schematic representation of a coronal section through the rat hippocampal formation. The tracer injection target site (dotted ellipse drawn to approximate scale) is aimed at distal area CA3 and overlaps with the mossy fiber pathway as indicated. c, dentate crest; CA1–CA3, Ammon's horn pyramidal fields; DGs, suprapyramidal blade of the dentate gyrus; DGi, infrapyramidal blade; gcl, granule cell layer; h, hilus; hf, hippocampal fissure; lv, lateral ventricle; sgz, subgranular zone. Adapted from Swanson (1992) with permission from Elsevier Science.

MATERIALS AND METHODS

Animal care and treatments

Adult male Sprague-Dawley rats (350–450 g; Charles River Laboratories, Wilmington, MA) were used as experimental subjects. Animals were maintained on a 12:12 light:dark cycle (lights off 18:00 hours) and were provided with unlimited access to food and water. All animal experimentation was conducted in accordance with University guidelines and with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Rats were injected with 5′-bromo-2′-deoxyuridine (BrdU; 200 mg/kg in saline containing 0.007 N NaOH, i.p.), a marker of proliferating cells and their progeny (Nowakowski et al., 1989). Twenty-four hours (n = 4 injections), 1 week (n = 6 injections), 2 weeks (n = 8 injections), or 3 weeks (n = 8 injections) after BrdU injection, rats were anesthetized with sodium pentobarbital (Nembutal; 50 mg/kg body weight, i.p.) and stereotaxically injected with the retrograde tracers Fast blue (FB; 200 nl of a 2.0% solution in sterile isotonic saline; Sigma, St. Louis, MO) or FluoroRuby (FR; 200 nl of a 10.0% solution in sterile isotonic saline; Molecular Probes, Eugene, OR) into the distal CA3 hippocampal region (stereotaxic coordinates in mm; anteroposterior, −3.3; lateromedial, ± 3.6; dorsoventral, −3.1; Paxinos and Watson, 1986; see Fig. 1). The 24-hour time point was selected, because it is sufficient for the completion of one cell cycle by BrdU-labeled cells (Nowakowski et al., 1989) but not for incorporation into the gcl or for the expression of biochemical markers of mature neurons (Cameron et al., 1993). The 1-, 2-, and 3-week post-BrdU survival times were chosen to coincide with changes in number, morphology, position, and the expression of biochemical markers by BrdU-labeled cells that may be characteristic of different stages of neuronal maturity within this system. Adult-generated hippocampal neurons express markers of immature neurons, such as TOAD-64, by 1 week after BrdU labeling but do not begin to express mature neuronal characteristics until between 2 and 3 weeks after their genesis (Cameron et al., 1993; Gould et al., 1999a).

Bilateral injections were performed by using a different tracer (FB or FR) in each hemisphere. Because mossy fiber projections are ipsilateral (Blackstad et al., 1970; Swanson et al., 1978), each injection site and its associated gcl labeling were examined as a separate case. Animals were deeply anesthetized with Nembutal (100 mg/kg body weight, i.p.) 3 days after stereotaxic surgery and transcardially perfused with 4.0% paraformaldehyde in isotonic phosphate-buffered saline, pH 7.4, at 2°C. The brains were then removed from the cranium and postfixed for 3 days in fresh perfusion medium at 4°C prior to processing for BrdU immunohistochemistry.

The 3-day post-tracer injection recovery period was sufficient to allow the transport of tracer from the CA3 injection site to even the most distal regions of the gcl. It is possible, however, that some BrdU-labeled cells, particularly those in sections coincident with the injection site, may have incorporated FB or FR within hours of tracer administration. The postoperative recovery period, therefore, introduces a 3-day range in which tracer uptake by BrdU-labeled cells is possible: we refer to the experimental groups by the upper limit of this range at 4 days, 10 days, 17 days, and 24 days after BrdU injection, respectively.

BrdU immunohistochemistry

Forty-micrometer coronal sections through the dentate gyrus were cut and collected into a bath of 0.1 M phosphate buffered saline, pH 7.4, by using an oscillating tissue slicer. A one-in-five series of sections was then mounted onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA), washed free of salts, and dried. A hydrophobic barrier was applied to the edge of each slide (PAP-pen; EMS, Fort Washington, PA) prior to rehydration in Tris-buffered saline (TBS; 100 mM Tris-HCl, 0.9% NaCl, pH 7.6, at room temperature). Unless otherwise noted, all subsequent incubations were performed in TBS. Sections were trypsinized (100 μg/ml in 0.1 M Tris-HCl, pH 7.4, and 0.1% $CaCl_2$) for 10 minutes, incubated in 2 N HCl for 30 minutes, rinsed, and incubated for 60 minutes with 3.0% normal horse serum (NHS; Vector Laboratories, Burlingame, CA). The NHS was eluted prior to application of anti-BrdU monoclonal antisera (Novocastra, Newcastle upon Tyne, United Kingdom: diluted 1:100 plus 0.5% Tween-20 in TBS, pH 8.2). Sections were incubated for 2 hours, rinsed, and incubated in biotinylated anti-mouse immunoglobulin

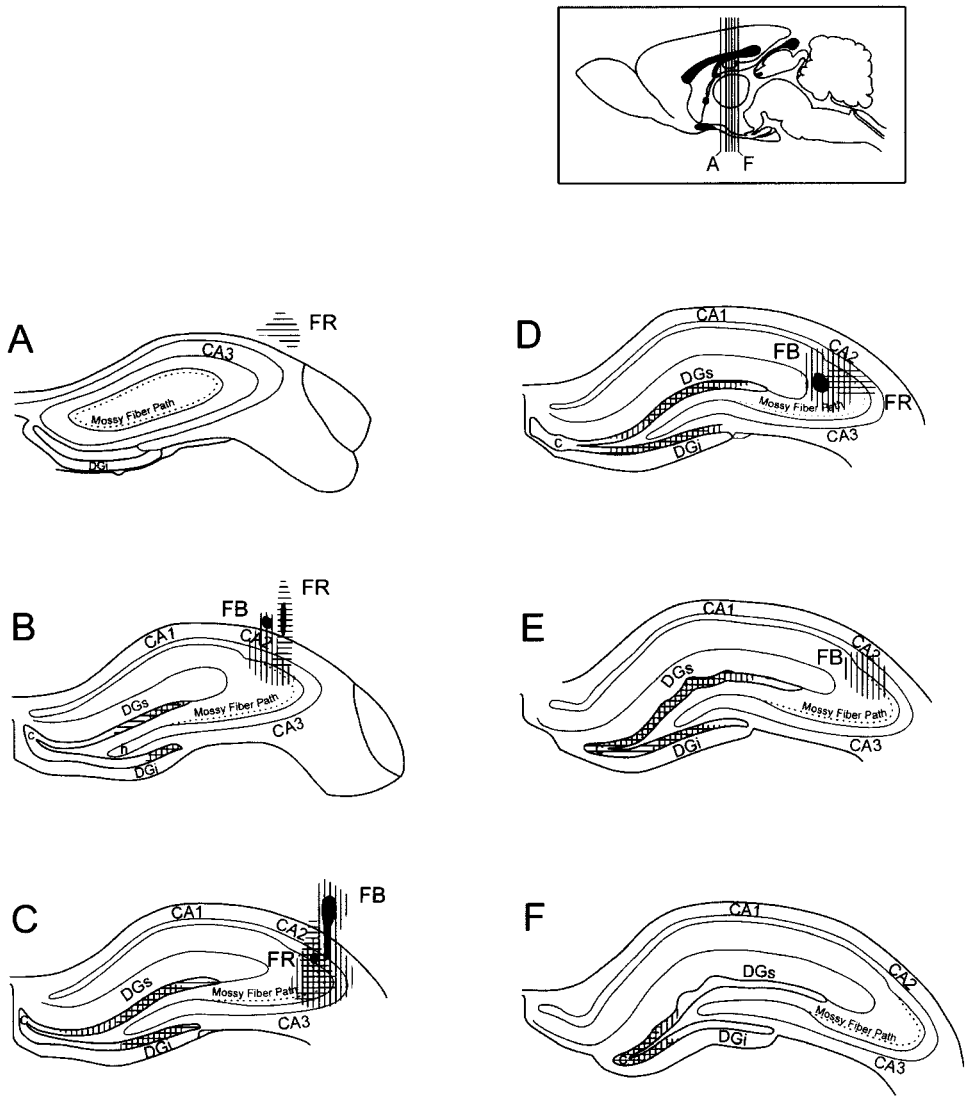

Fig. 2. **A–F:** Representative injection sites resulting from the stereotaxic infusion of the retrograde tracer Fast blue (FB; vertical hatching) or FluoroRuby (FR; horizontal hatching) into hippocampal area CA3 and the mossy fiber path of two different animals. FB and FR injections resulted in a similar degree of labeling of the ipsilateral dentate gyrus. The areas of labeling resulting from these injections are represented by vertical or horizontal hatching in the granule cell layer (gcl). Note that significant labeling is evident in F, posterior to the caudalmost border of the injection site. Both of these injections were performed 3 weeks after 5′-bromo-2′-deoxyuridine (BrdU) administration. Black portions represent tissue necrosis associated with the needle track. **Inset:** Schematic sagittal section of the rat brain depicting the anteroposterior levels of the coronal sections shown in A–F. Drawings adapted from Swanson (1992) with permission from Elsevier Science. For abbreviations, see Figure 1.

G (IgG) antisera (1:200) containing 1.5% NHS for 1 hour. Sections were rinsed and incubated in avidin-conjugated CY-2 fluorochrome (1:1,000) for 30 minutes, rinsed, dried, and coverslipped under 70% glycerol:TBS prior to observation.

Data analysis

Prior to analysis, all slides were coded, and the code was not broken until data collection was complete. Each dentate gyrus was examined and included for analysis if it was found that the effective tracer-uptake zone, indicated by the area of tracer-labeled glia, included the CA3 region and if tracer injection resulted in retrograde transport and labeling of the gcl (see Fig. 2). The following experimental group sizes were obtained for analysis, with the remaining injections (those outside of the CA3 region) examined as controls (see Fig. 3): 4 days (n = 3 injection sites), 10 days (n = 6 injection sites), 17 days (n = 7 injection sites), and 24 days (n = 6 injection sites).

The areas of effective tracer uptake and total sampling volume were measured for each valid injection by using Cavalieri's principle (Gundersen et al., 1988) and Image Pro software. Cell counts of the total number of BrdU-labeled cells and those incorporating retrograde tracer were obtained only from FB-or FR-labeled regions of the gcl. Cells of obvious glial morphology (small with irregular or triangular-shaped cell bodies) were excluded from the counts. Cell counts were normalized to the sampling

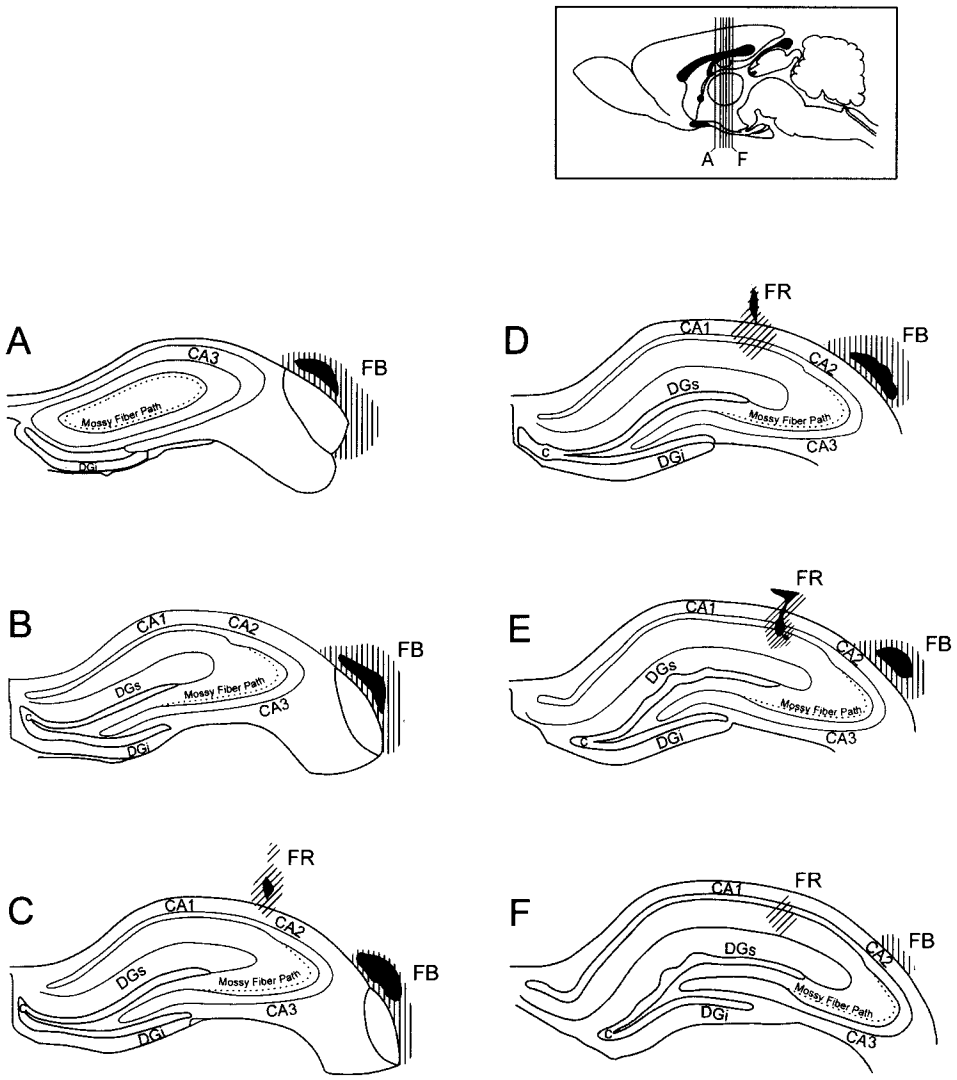

Fig. 3. **A–F:** Representative control injection sites resulting from the stereotaxic infusion of the retrograde tracer FB (vertical hatching) or FluoroRuby FR (diagonal hatching) into brain regions close to but not overlapping with hippocampal area CA3 and the mossy fiber path. Neither injection resulted in detectable labeling of the ipsilateral dentate gyrus. Black portions represent tissue necrosis associated with the injection. **Inset:** Schematic sagittal section of the rat brain depicting the anteroposterior levels of the coronal sections shown in A–F. Drawings adapted from Swanson (1992) with permission from Elsevier Science. For abbreviations, see Figure 1.

volume. All quantitative analysis was performed by using an Olympus BX-60 fluorescent microscope (Olympus, Tokyo, Japan). Colabeling of BrdU and tracer was verified by using a Zeiss Axiovert confocal laser scanning microscope (510LSM; Zeiss, Thornwood, NY).

The area of effective tracer uptake and volume of gcl labeling were analyzed simultaneously as functions of 1) post-BrdU injection survival time and 2) retrograde tracer using parametric multivariate analyses of covariance. Multivariate F-test statistics (Wilk's lambda) are reported ($\alpha = 0.05$). The total numbers of BrdU-labeled cells and the numbers of tracer-labeled, BrdU-positive cells were expressed per unit sampling volume prior to analysis. These values, as well as cell percentages, were analyzed by using distribution-free methods: the Kruskal-Wallis analysis of variance by ranks ($\alpha = 0.05$; H statistic reported) followed by planned, pairwise comparisons by using the Mann-Whitney U-test.

RESULTS

Location, morphology, and numbers of adult-generated cells

At all time points, BrdU-labeled cells were observed in the dentate gyrus. Changes in the location, morphology, and numbers of these cells were observed over time. With increasing time after BrdU injection, proportionately more labeled cells were located in the gcl compared with the sgz and hilus. Moreover, at the later time points (17 days and 24 days), more BrdU-labeled cells had the morphology of mature granule cells, i.e., the nuclei were relatively large and round. The maximal number of BrdU-labeled cells was observed in the gcl 10 days after BrdU administration. At this point, most cells were closer in size to neighboring mature granule cells but typically had nuclei that were oval or elongated. Between 10 days and 17 days, a decrease

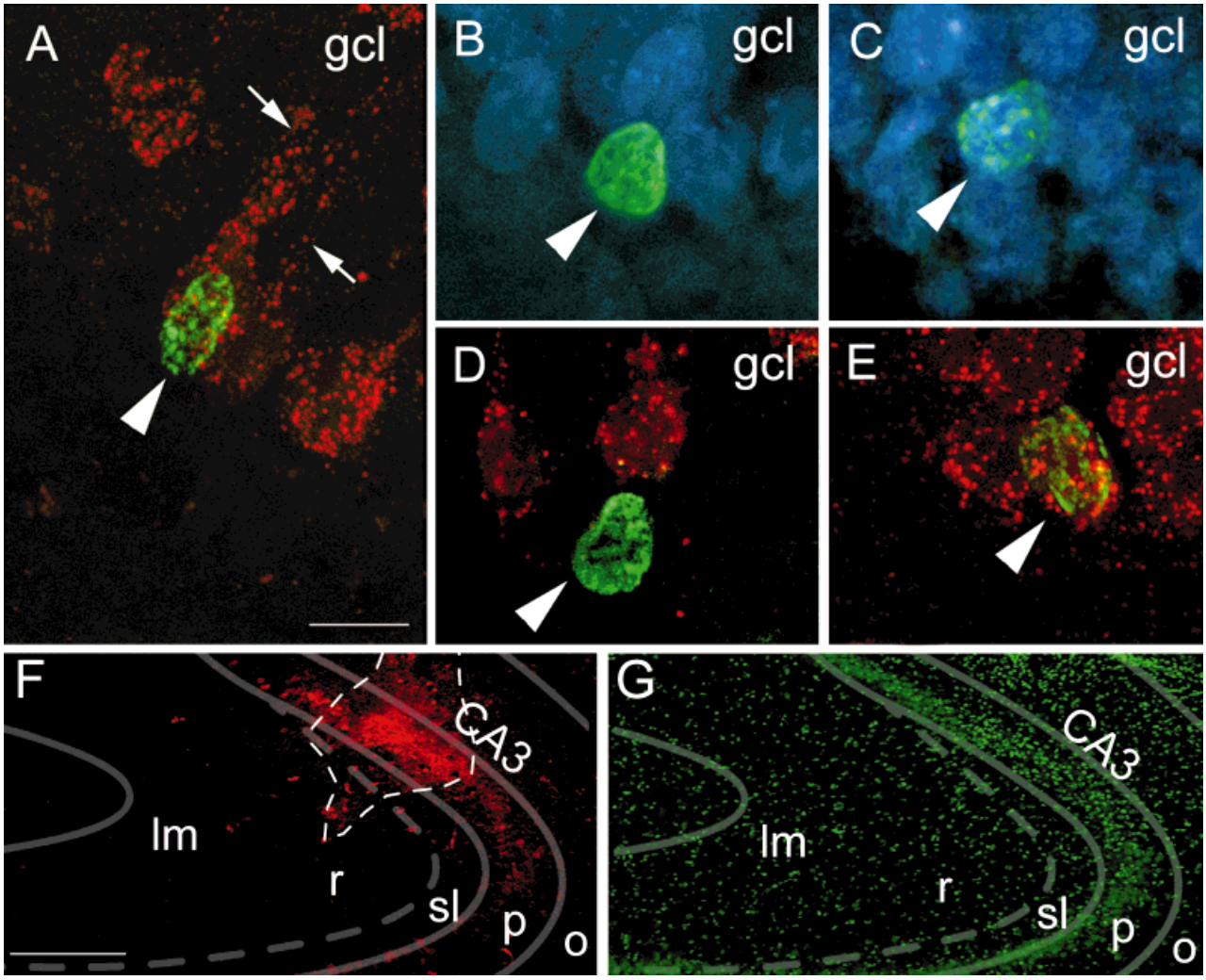

Fig. 4. Color images produced from captured digital confocal laser scanning microscopy by using Adobe Photoshop software (Adobe Systems, Seattle, WA) showing BrdU-labeled cells (green) residing in the gcl of adult male rats that were injected in the CA3 region with either FB (blue nuclei and cytoplasm) or FR (orange-red cytoplasm and processes). **A:** Immature neuron retrogradely labeled with FR (arrowhead) 10 days after BrdU administration. This cell has characteristic immature nuclear morphology but extends a dendritic process (arrows). Contrast the somewhat elongated nucleus of this cell to the more rounded appearance of the BrdU-labeled nuclei from later time points depicted in B–D. **B:** Nonfilled, BrdU-labeled cell (arrowhead) located near granule neurons that was labeled retrogradely with FB (blue). **C:** BrdU-labeled neuron that also was labeled retrogradely with FB (arrowhead). Both of the labeled cells in B and C reside in the gcl of the same animal that was perfused 24 days after BrdU administration. **D:** Nonfilled, BrdU-labeled cell (arrowhead). **E:** BrdU-labeled neuron (arrowhead) that also was labeled retrogradely with FR. Both of the labeled cells in D and E reside in the gcl of the same animal that was perfused 17 days after BrdU administration. **F:** Low-power (×7) confocal image of a representative FR injection site. The effective uptake zone is indicated by the white dashed outline and is centered in the pyramidal cell layer (p) of distal CA3. Note that the injection site does not encroach on the dentate gyrus. **G:** The same section shown in F counterstained with the fluorescent DNA dye YOYO-1 (Molecular Probes, Eugene, OR) to reveal anatomic features near the injection site. CA3, region CA3 of Ammon's horn; lm, stratum lacunosum-moleculare; o, stratum oriens; p, pyramidal layer; r, stratum radiatum; sl, stratum lucidum. Scale bars = 10 μm in A (also applies to B–D); 250 μm in F (also applies to G).

in the number of BrdU-labeled cells was observed, but no further decrease in cell number was seen between 17 days and 24 days. By 17 days, very few BrdU-labeled cells were observed in the hilus or sgz.

Retrograde tracer injections

FB and FR injection sites consisted of two concentric fluorescent zones. A small, bright, and dense zone immediately subadjacent to the termination of the needle tract was surrounded by a broader, more diffuse area that expanded radially for a variable distance. Within both fluorescent zones, tracer appeared to label both neurons and glia; thus, the edge of the larger, diffuse zone was considered to circumscribe the area of effective tracer uptake. Although FB appeared to diffuse at the injection site to a greater extent compared with FR, neither survival time after BrdU injection ($F[2,13] = 0.73$; $P = 0.13$) nor retrograde tracer ($F[6,26] = 0.70$; $P = 0.55$; Fig. 2) was a significant factor in determining the area of effective tracer uptake or volume of the gcl that was labeled with tracer. None of the selected dye injection sites labeled the gcl directly (Fig. 4F,G).

TABLE 1. Densities of Bromodeoxyuridine-Labeled Cells and Percentages and Densities of Tracer-Filled, Bromodeoxyuridine-Labeled Cells in the Granule Cell Layer of Adult Male Rats at Various Times After Bromodeoxyuridine Administration[1]

Group	No.	Density of BrdU-labeled cells (cells/mm^3)	Tracer-filled, BrdU-labeled cells (cells/mm^3)	Percentage of tracer-filled, BrdU-labeled cells
4 day	3	307 ± 121.3*	0 ± 0.0*	0*
10 day	6	709 ± 92.6**	65 ± 16.0**	8.9*
17 day	7	294 ± 48.4*	128 ± 22.0***	45.8**
24 day	6	269 ± 42.8*	117 ± 50.4***	47.6**

[1]Significant differences in the density of bromodeoxyuridine (BrdU)-labeled cells [H (3) = 10.98; $P = 0.01$]; the density of tracer-filled, BrdU-labeled cells [H (3) = 8.89; $P < 0.05$]; and the percentage of tracer-filled, BrdU-labeled cells [H (3) = 11.86; $P < 0.01$] were observed as a function of increasing survival time after BrdU injection. Values within each column designated by different numbers of asterisks are statistically different from one another ($P < 0.05$; Mann-Whitney U-test).

With both FB and FR, retrograde transport to levels located caudal to the injection site was observed often (Fig. 2). At levels coincident with or slightly rostral to the injection site, the laterosuperior tips of both the suprapyramidal and infrapyramidal blade of the dentate gyrus gcl usually were labeled retrogradely with tracer. The area of tracer incorporation proceeded to fill successively medial levels of both blades at progressively caudal levels relative to the injection site and, at the most posterior levels at which tracer could be detected, was observed in the crest of the dentate gyrus. FB was localized primarily to the granule cell body, whereas FR provided punctate labeling of the granule cell body and, to a variable extent, was localized to proximal dendrites and to smaller caliber, studded axonal processes.

Colocalization of BrdU and retrograde tracer in the gcl

In the animals that received tracer injections into CA3 24 hours after BrdU administration and were perfused 3 days later, we did not observe any FB- or FR-labeled, BrdU-positive cells. The earliest point at which we observed BrdU-labeled cells colocalized with retrograde tracer was in the 10-day group, indicating that the axons of these neurons had reached CA3 between 4 days and 10 days after mitosis (Fig. 4A). These cells typically were oval or elongated, were large relative to BrdU-labeled cells that were not labeled with tracer at this time point, and were located in the gcl, not in the sgz or the hilus. However, although many of these cells were observed to extend dendritic processes, they clearly had not yet attained the distinct nuclear morphology of neighboring mature granule cells, which tended to be somewhat larger and were round, not oval.

The percentage of adult-generated granule cells labeled with retrograde tracer increased with increasing survival time after BrdU injection (see Table 1, Fig. 4C,E), although, at all time points examined, we observed BrdU-labeled granule cells that were not double labeled with FB or FR, despite being located in gcl regions that were heavily labeled with tracer (Fig. 4B,D). Between 10 days and 17 days after BrdU labeling, the percentage of adult-generated granule cells retrogradely labeled with FB or FR increased from 9% to ≈ 46%. This increase appears to reflect both an overall increase in the numbers of BrdU-labeled cells that were colabeled with tracer and a substantial reduction in the total numbers of BrdU-labeled cells in the dentate gcl. We did not observe any tracer-labeled, BrdU-positive cells outside of the gcl.

We often observed cells labeled with both BrdU and retrograde tracer on sections posterior to the injection site. Particularly at later time points, 17 days or 24 days after the administration of BrdU, tracer-labeled, adult-generated neurons could be found as far as 1 mm caudal to the injection site (Fig. 5).

DISCUSSION

Consistent with previous studies (Cameron et al., 1993; Gould et al., 1999a), we observed characteristic changes in the numbers, distribution, and morphology of BrdU-labeled cells with increasing post-BrdU survival times. The numbers of adult-generated cells in the dentate gyrus increased between 4 days and 10 days after mitosis and had declined by the 17-day time point. At early time points after BrdU injection (4 days and 10 days), many labeled cells were located in the hilus or the sgz and exhibited smaller and nonround nuclei. By 17 days, the majority of BrdU-labeled cells were located within the gcl and had relatively large, round or oval nuclei. By 24 days, BrdU-labeled cells were similar in size and shape to granule neurons located in the superficial aspect of the gcl.

The absence of tracer-labeled, BrdU-positive cells in animals injected with either FB or FR 24 hours after BrdU administration suggests that adult-generated neurons between 1 day and 4 days of age have either not extended axonal processes or their axons have not yet extended into the region of the injection site. FB and FR could be detected first in BrdU-labeled cells in the 10-day group, indicating that new cells have axons in CA3 some time between 4 days and 10 days after mitosis. These cells exhibited oval or elongated nuclei, were always located within the gcl, but clearly had not yet attained the distinct morphology characteristic of surrounding mature granule neurons. At later time points, 17 days and 24 days after BrdU administration, more BrdU-positive cells were labeled retrogradely with tracer. These were largely indistinguishable in size and nuclear morphology from neighboring granule neurons. Moreover, at these later time points, double-labeled cells were detected up to 1 mm posterior to the tracer injection site. If the axons of adult-generated granule cells follow trajectories similar to those of mossy fibers in general, traveling within the transverse axis at coronal levels coincident with their site of origin before diving into the longitudinal axis near CA2, then it is likely that these axons are much longer than 1 mm (see also Stanfield and Trice, 1988).

We did not observe large, round, BrdU-positive cells in any area other than the gcl, nor did we observe tracer-labeled, BrdU-positive cells in the hilus or sgz. This may indicate that adult-generated cells do not contribute to the ectopic mossy fiber projection pathway previously reported by Gaarskjaer and Laurberg (1983). Alternatively, our postinjection survival times or tracer placement may have been inadequate for the detection of this class of projection.

Our results suggest that the axons of adult-generated granule neurons are present in area CA3 between 4 days and 10 days after BrdU labeling. This corresponds to a time at which adult-generated granule cells have not yet attained mature morphologic or biochemical characteristics (Cameron et al., 1993). Studies of hippocampal slice explants (Dailey et al., 1994) and cell cultures (Basarsky et al., 1994) and studies of the developing mossy fiber system

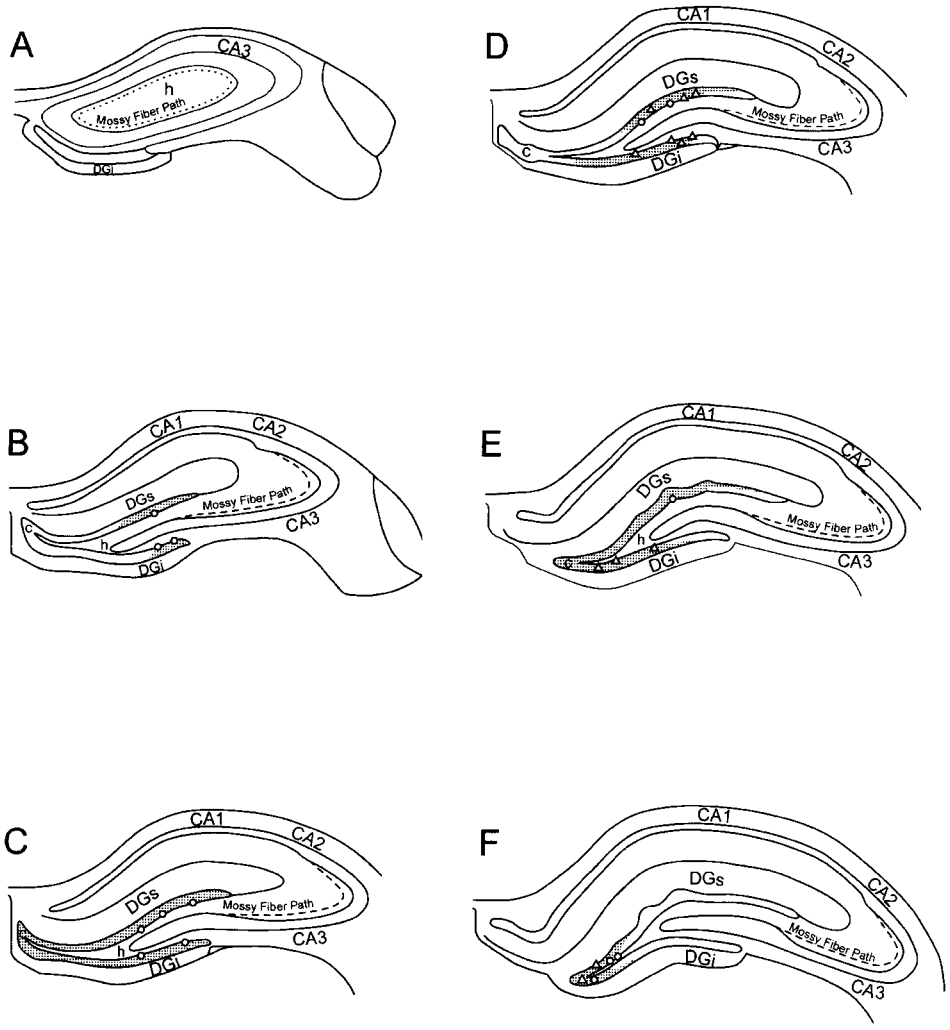

Fig. 5. **A–E:** Schematic representation of BrdU-labeled cells (circles) and tracer-labeled, BrdU-positive cells (triangles) located within the dentate gyrus 3 weeks after BrdU injection. FB injection case was presented in Figure 2. BrdU-positive cells in regions of the gcl that were not labeled with tracer are not shown in these drawings, because these cells were not included in the analysis. Shaded areas indicate the areas of the gcl that were retrogradely labeled by the FB injection. A–F correspond to the anteroposterior levels depicted in the insets of Figures 2 and 3. Note in particular the presence of tracer-labeled, BrdU-positive cells in F at a level well posterior to the caudalmost border of the injection site. For abbreviations, see Figure 1.

in vivo (Vijayan, 1986) indicate that synapse formation rapidly follows the extension of incipient granule cell axons into area CA3. This raises the possibility that the axons of immature adult-generated granule cells also may form functional synapses.

Methodological considerations

It is likely that BrdU-labeled cells incorporate tracer by means of retrograde transport from CA3 as opposed to nonspecific mechanisms of tracer uptake, for example, diffusion from the injection site or, after transport, from adjacent tracer-labeled cells in the gcl (Bentivoglio et al., 1980). First, BrdU-labeled cells did not incorporate FB or FR when the tracer was injected 24 hours after BrdU administration. Second, at other time points, BrdU-labeled cells adjacent to FB- and FR-labeled granule cells often were not colabeled with tracer.

It is likely that the percentages and densities of BrdU-labeled cells we report as colabeled with retrograde tracer are underestimates of the proportion of new cells that actually extend axons into the CA3 region for two reasons. First, BrdU-labeled cells may have axons in parts of the CA3 region that were not injected with tracer. Because the discrete tracer injections performed did not label the entire CA3 region, substantial numbers of BrdU-labeled cells with axons outside of the areas of effective tracer uptake would not be labeled with FB or FR. Although, in development, granule neurons that reside in proximity within the gcl project to the same region of CA3 (Gaarskjaer, 1986), it is not known whether this also occurs in adulthood. Second, new cells may project to other mossy fiber target sites, such as hilar mossy cells (Frotscher et al., 1994).

Technical caveats aside, comparisons across time points suggest an increase in the number and percentage of adult-generated cells that extend axons into hippocampal area CA3 with increasing time after BrdU incorporation. This occurs in conjunction with a decline in the total number of BrdU-labeled cells. Perhaps the extension of

axons into appropriate hippocampal target fields serves as a survival cue at a time when many adult-generated cells are dying. The formation of synapses, or even the proximity of new afferents to their target neurons, may provide a means by which cell contact and/or paracrine signaling facilitate neuronal survival (de la Cruz et al., 1996; Goodman and Tessier-Lavigne, 1997). Alternatively, the formation of functional mossy fiber synapses may allow mechanisms, such as activity-dependent synaptic strengthening, to promote selectively the survival of immature adult-generated neurons.

Projection patterns of adult-generated granule cells

Neuron production in most regions of the mammalian central nervous system is a temporally discrete process, ending prior to parturition. In contrast, granule cell production in the rat begins embryonically, peaks shortly after birth, and continues at lower levels throughout adulthood (Bayer,1980a,b; Schlessinger et al., 1975; Cameron et al., 1993).

Four organizational gradients are recognized in the formation of the rodent gcl (Angevine 1965; Schlessinger et al., 1975; Bayer,1980a,b): 1) Granule cells are integrated into the gcl in a cell-packing or outside-in manner; 2) the suprapyramidal blade forms before birth, whereas the infrapyramidal blade is formed postnatally; 3) maturation of the dentate gyrus proceeds in a temporal-to-septal direction; and 4) the addition of granule cells occurs in a lateral-to-medial direction with respect to the transverse axis. Retrograde tracer injections into distal CA3 reveal a distinct pattern of labeling likely to reflect the aforementioned developmental patterns of granule cell production and integration into the gcl. For example, the laterosuperior aspect of the suprapyramidal dentate blade is labeled at levels anterior and, particularly, posterior to the tracer injection site compared with the medioinferior region or the dentate crest, which takes up tracer at levels approximately coincident with the injection site (Gaarskjaer, 1981). These results have been interpreted to indicate wider septotemporal axon divergence for developmentally older granule cells and, mechanistically, have been posited to reflect increased competition for axonal termination sites as development proceeds (Blackstad et al., 1970). However, in the context of these studies, it is important to note that "axonal divergence" can be interpreted only from the perspective of neuronal populations and not from that of individual granule cells. Single retrograde tracer injections, for example, cannot distinguish between the projections of two granule neurons versus the divergent projection of a single neuron. Nonetheless, axonal divergence at the regional or single-cell level may have important biological consequences. Knowledge of the axonal arborization patterns of adult-generated granule neurons may provide valuable insight into their contribution to normal hippocampal function. From a developmental standpoint, it may be predicted that the axonal divergence of adult-generated granule neurons should be restricted relative to perinatally derived granule cells, because they are much younger. However, additional work will be required to determine the extent to which the mossy fibers of individual adult-generated granule neurons diverge in their projection to the hippocampus.

Functional significance of adult-generated cells

Although it has been recognized previously that phenotypically mature, adult-generated hippocampal neurons extend axons into the mossy fiber pathway in the rat, the present study indicates that this contribution is far more significant than previously reported. In addition, we have found that immature granule cells also extend mossy fibers into area CA3, the significance of which remains unclear. Adult-generated granule cells may pass through a period of functional immaturity similar to the perinatal developmental period. During development, immature granule neurons exhibit giant depolarizing action potentials in response to stimulation of the γ-aminobutyric acid A receptor (Liu et al., 1996; Hollrigel et al., 1998) and prolonged long-term potentiation at the perforant path synapse (Bronzino et al., 1994). These phenomena may influence neuronal differentiation, for example, by mediating intracellular calcium flux (Ben-Ari et al., 1997), and, in addition, may contribute to the overall network properties of the hippocampal formation. The unique physiological attributes of immature granule neurons in development, coupled with the present evidence that these cells rapidly extend axons into area CA3, imply the presence of a functionally heterogeneous population of granule neurons within the dentate gyrus. Perhaps these varied cell populations each provide unique input to the CA3 region.

Of some relevance to this idea is the identification of a sensitive period during which experience influences the survival of immature adult-generated neurons in the male rat (Gould et al., 1999a). Typically in this species, and under standard laboratory conditions, a large proportion of adult-generated neurons degenerate during a period that follows 1–2 weeks after BrdU labeling (Cameron et al., 1993; Gould et al., 1999a). Spatial navigation learning in the Morris water maze and training in the trace form of classical eye-blink conditioning, paradigms that specifically require the hippocampus (Morris, 1984; LaBar and Disterhoft, 1998), have been demonstrated to increase the survival of adult-generated neurons during this sensitive period (Gould et al., 1999a). The present study presents the possibility that immature neuronal networks may provide the foundation for learning-dependent neuronal rescue, perhaps by forming an anatomic substrate for activity-dependent synaptic strengthening.

ACKNOWLEDGMENTS

We gratefully acknowledge Patrick Tanapat for helpful comments in the manuscript and Joseph Goodhouse for assistance with confocal imaging. This work was supported by National Institutes of Health individual NRSA fellowship MH12362 and grants MH52423 and MH59740.

LITERATURE CITED

Altman J, Das GD. 1967. Postnatal neurogenesis in the guinea pig. Nature 214:1098–1101.

Angevine JB. 1965. Time of neuron origin in the hippocampal region. An autoradiographic study in the mouse. Exp Neurol Suppl 2:1–70.

Basarsky TA, Parpura V, Haydon PG. 1994. Hippocampal synaptogenesis in cell culture: developmental time course of synapse formation, calcium influx, and synaptic protein distribution. J Neurosci 14:6402–6411.

Bayer SA. 1980a. Development of the hippocampal region in the rat. I. Neurogenesis examined with 3H-thymidine autoradiography. J Comp Neurol 190:87–114.

Bayer SA. 1980b. Development of the hippocampal region in the rat. II. Morphogenesis during embryonic and early postnatal life. J Comp Neurol 190:115–134.

Ben-Ari Y, Khazipov R, Leinekugel X, Caillard O, Gaiarsa JL. 1997. GABAA, NMDA and AMPA receptors: a developmentally regulated "menage à trois". Trends Neurosci 20:523–529.

Bentivoglio M, Kuypers HG, Catsman-Berrevoets CE, Loewe H, Dann O. 1980. Two new fluorescent retrograde neuronal tracers which are transported over long distances. Neurosci Lett 18:25–30.

Blackstad TW, Brink K, Hem J, Jeune B. 1970. Distribution of hippocampal mossy fibers in the rat. An experimental study with silver impregnation methods. J Comp Neurol 138:433–449.

Bronzino JD, Abu-Hasaballah K, Austin-LaFrance RJ, Morgane PJ. 1994. Maturation of long-term potentiation in the hippocampal dentate gyrus of the freely moving rat. Hippocampus 4:439–446.

Cameron HA, Woolley CS, McEwen BS, Gould E. 1993. Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. Neuroscience 56:337–344.

Dailey ME, Buchanan J, Bergles DE, Smith SJ. 1994. Mossy fiber growth and synaptogenesis in rat hippocampal slices in vitro. J Neurosci 14:1060–1078.

de la Cruz RR, Pastor AM, Delgado-Garcia JM. 1996. Influence of the postsynaptic target on the functional properties of neurons in the adult mammalian central nervous system. Rev Neurosci 7:115–149.

Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH. 1998. Neurogenesis in the adult human hippocampus. Nat Med 4:1313–1317.

Frotscher M, Soriano E, Misgeld U. 1994. Divergence of hippocampal mossy fibers. Synapse 16:148–160.

Gaarskjaer FB. 1981. The hippocampal mossy fiber system of the rat studied with retrograde tracing techniques. Correlation between topographic organization and neurogenetic gradients. J Comp Neurol 203:717–735.

Gaarskjaer FB. 1986. The organization and development of the hippocampal mossy fiber system. Brain Res 396:335–357.

Gaarskjaer FB, Laurberg S. 1983. Ectopic granule cells of hilus fasciae dentatae projecting to the ipsilateral regio inferior of the rat hippocampus. Brain Res 274:11–16.

Goodman CS, Tessier-Lavigne M. 1997. Molecular mechanisms of axon guidance and target recognition. In: Cowan WM, Jessell TM, Zipursky SL, editors. Molecular and cellular approaches to neural development. Oxford: Oxford University Press. p 108–178.

Gould E, Tanapat P. 1997. Lesion-induced proliferation of neuronal progenitors in the dentate gyrus of the adult rat. Neuroscience 80:427–436.

Gould E, McEwen BS, Tanapat P, Galea LAM, Fuchs E. 1997. Neurogenesis in the dentate gyrus of the adult tree shrew is regulated by psychosocial stress and NMDA receptor activation. J Neurosci 17:2492–2498.

Gould E, Tanapat P, McEwen BS, Flugge G, Fuchs E. 1998. Proliferation of granule cell precursors in the dentate gyrus of adult monkeys is diminished by stress. Proc Natl Acad Sci USA 95:3168–3171.

Gould E, Beylin AV, Tanapat P, Reeves AJ, Shors TJ. 1999a. Learning enhances adult neurogenesis in the hippocampal formation. Nature Neurosci 2:260–265.

Gould E, Reeves AJ, Fallah M, Tanapat P, Gross CG, Fuchs E. 1999b. Hippocampal neurogenesis in adult Old World primates. Proc Natl Acad Sci USA 96:5263–5267.

Gundersen HJ, Bagger P, Bendtsen TF, Evans SM, Korbo L, Marcussen N, Moller A, Nielsen K, Nyengaard JR, Pakkenberg B. 1988. The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. APMIS 96:857–881.

Hollrigel GS, Ross ST, Soltesz I. 1998. Temporal patterns and depolarizing actions of spontaneous GABAA receptor activation in granule cells of the early postnatal dentate gyrus. J Neurophysiol 80:2340–2351.

Kaplan MS, Bell DH. 1984. Mitotic neuroblasts in the 9-day-old and 11-month-old rodent hippocampus. J Neurosci 4:1429–1441.

Kaplan MS, Hinds JW. 1977. Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. Science 197:1092–1094.

Kempermann G, Kuhn HG, Gage FH. 1997. More hippocampal neurons in adult mice living in an enriched environment. Nature 386:493–495.

LaBar KS, Disterhoft JF. 1998. Conditioning, awareness, and the hippocampus. Hippocampus 8:620–626.

Liu YB, Lio PA, Pasternak JF, Trommer BL. 1996. Developmental changes in membrane properties and postsynaptic currents of granule cells in rat dentate gyrus. J Neurophysiol 76:1074–1088.

Markakis E, Gage FH. 1999. Adult-generated neurons in the dentate gyrus send axonal projections to field CA3 and are surrounded by synaptic vesicles. J Comp Neurol 406:449–460.

Morris R. 1984. Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods 11:47–60.

Nowakowski RS, Lewin SB, Miller MW. 1989. Bromodeoxyuridine immunohistochemical determination of the lengths of the cell cycle and the DNA-synthetic phase for an anatomically defined population. J Neurocytol 18:311–318.

Parent JM, Yu TW, Leibowitz RT, Geschwind DH, Sloviter RS, Lowenstein DH. 1997. Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. J Neurosci 17:3727–3738.

Paxinos G, Watson C. 1986. The rat brain in stereotaxic coordinates. New York: Academic Press.

Schlessinger AR, Cowan WM, Gottlieb DI. 1975. An autoradiographic study of the time of origin and the pattern of granule cell migration in the dentate gyrus of the rat. J Comp Neurol 159:149–175.

Scott BW, Wang S, Burnham WM, De Boni U, Wojtowicz JM. 1998. Kindling-induced neurogenesis in the dentate gyrus of the rat. Neurosci Lett 248:73–76.

Seki T, Arai Y. 1995. Age-related production of new granule cells in the adult dentate gyrus. Neuroreport 6:2479–2482.

Stanfield BB, Trice JE. 1988. Evidence that granule cells generated in the dentate gyrus of adult rats extend axonal projections. Exp Brain Res 72:399–406.

Swanson LW, Wyss JM, Cowan WM. 1978. An autoradiographic study of the organization of intrahippocampal association pathways in the rat. J Comp Neurol 181:681–715.

Swanson LW. 1992. Brain maps: structure of the rat brain. Amsterdam: Elsevier Science.

Vijayan VK. 1986. Morphogenesis of the mossy fiber synapses in the hippocampus of the rhesus monkey. Brain Res 390:259–270.