Differential Properties of Dentate Gyrus and CA1 Neural Precursors

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ABSTRACT: In the present article we investigated the properties of CA1 and dentate gyrus cell precursors in adult rodents both *in vivo* and *in vitro*. Cell proliferation *in situ* was investigated by rating the number of cells incorporating BrdU after kainate-induced seizures. CA1 precursors displayed a greater proliferation capacity than dentate gyrus precursors. The majority of BrdU-labeled cells in CA1 expressed Nestin and Mash-1, two markers of neural precursors. BrdU-positive cells in the dentate gyrus expressed Nestin, but only a few expressed Mash-1. In animals pretreated with the antimitotic azacytidine, the capacity of kainate to enhance the proliferation was higher in CA1 than in the dentate gyrus. Differences in intrinsic progenitor cell activity could underlie these different expansion capacities. Thus, we compared the renewal- expansion and multipotency of dentate gyrus and CA1 precursors isolated *in vitro*. We found that the dissected CA1 region, including the periventricular zone, is enriched in neurosphere-forming cells (presumed stem cells), which respond to either EGF or FGF-2. Dentate gyrus contains fewer neurosphere-forming cells and none that respond to FGF-2 alone. Neurospheres generated from CA1 were multipotent and produced neurons, astrocytes, and oligodendrocytes, while dentate gyrus neurospheres mostly produced glial cells. The analysis of the effects of EGF on organotypic cultures of hippocampal slices depicted similar features: BrdU and Nestin immunoreactivities increased after EGF treatment in CA1 but not in the dentate gyrus. These results suggest that CA1 precursors are more stem-cell-like than granule cell precursors, which may represent a more restricted precursor cell. © 2004 Wiley Periodicals, Inc. J Neurobiol 62: 243–261, 2005

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INTRODUCTION

One of the more mitotically active zones in adult CNS is the dentate gyrus (DG), where granule cells continue to be generated throughout life in rodents (Altman and Das, 1965), macaques (Kornack and Rakic, 1999), and humans (Eriksson et al., 1998). The precise role for this sustained neurogenesis remains unclear, but, given its modulation by stress (Gould et al., 1997, 1998), exposure to an enriched environment (Kempermann et al., 1997, 1998), and training on associative learning tasks (Gould et al., 1999), granule cell proliferation may contribute to the hippocampal plasticity. Thus, it has been shown that the inhibition of neurogenesis results in a specific impairment of hippocampal-dependent trace conditioning (Shors et al., 2001). In addition, granule cell proliferation is highly increased in rodents after seizures (Parent et al., 1997) and ischemia (Takagi et al., 1999), and it has been suggested to contribute to the development of these. Though much attention has been paid to

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these events little is known about the possible mechanisms that are here implicated, and that may involve the blood brain barrier disruption, and the inflammation or the increased expression of trophic factors, including EGF (epidermal growth factor) and FGF-2 (fibroblast growth factor), the expression of which is increased after seizures (Ferrer et al., 1996; Bugra et al., 1994).

Neurogenesis in adult hippocampus may also include other hippocampal cell types, including pyramidal cells (Nakatomi et al., 2002) and calbindin-positive neurons in the mice CA1 field (Rietze et al., 2000), subgranular neurons of monkeys (Kornack and Rakic, 1999), and granulelike neurons in the hilus (Scharfman et al., 2002). These observations support the concept that a continuous renewal of neurons would contribute to plasticity and eventually help to maintain the integrity of neural networks in adult CNS (replacing dying cells). Interestingly, Nakatomi et al. (2002) have recently shown that CA1 precursors likely located within the periventricular zone generate pyramidal neurons in adult rats and may restore ischemia-induced CA1 damage, so confirming the stem cell nature of these progenitors. This article strongly supports hippocampal stem cells as a repairing tool for damaged brain.

Whether granule cells and CA1 precursors share similar properties in terms of expansion-renewal and multipotentiality is presently unclear. The nature of adult hippocampal precursors has been poorly identified. The observation that in the subgranular zone of adults not only neurons are generated but astrocytes and oligodendrocytes as well (Kornack and Rakic, 1999) supports the notion that they derive from a common multipotent precursor with stem cell properties (Gage et al., 1998). In fact, multipotent precursor cells, with high renewal-expansion capacities, have been isolated from adult hippocampus when cultured in the presence of either EGF+FGF-2 (Weiss et al., 1996a) or FGF-2 (Gage et al., 1998), factors known to be mitogenic for neural stem cells (reviewed in Weiss et al., 1996b). However, intracerebral application of EGF or FGF-2 alone failed to induce the proliferation of granule cells (Kuhn et al., 1997). It has been suggested that additional factors are required for the proliferation of granule cell precursors (Taupin et al., 2000). Alternatively, DG precursors would not really be stem cells. Interestingly, subgranular cells that express glial fibrillary acidic protein (GFAP) divide and generate new neurons under normal conditions, suggesting that astrocytes may be the precursors of granule cells in adult hippocampus (Seri et al., 2001). More recently, Seaberg and Van Der Kooy (2002) observed that DG precursors *in vitro* did not proliferate in the presence of EGF+FGF-2 and concluded that DG did not contain stem cells but restricted precursors. However, these authors did not exclude that other coadjuvant factors present in the tissue are required for their expansion (also see Taupin et al., 2000).

In the present article we investigated the properties of CA1 and DG precursors by analyzing their expansion-renewal capacities *in vivo* and *in vitro*.

METHODS

Animals' Treatment

To evaluate the capacity of hippocampal cell precursors to proliferate and generate neurons, young adult CD1 mice (40–50 g, 3 month old) were injected intraperitoneally (i.p.) with kainic acid (KA; 40 mg/kg; Sigma Aldrich, St Quentin Fallavier, France) dissolved in 0.2 μL of phosphate buffer (PB; pH 7.4). For subsequent experiments only the animals showing typical KA-induced limbic motor seizures for at least 2 h were used. Animals that had injection of vehicle alone were used as controls. One to thirty days after these treatments animals received a series of six i.p. (every 2 h) bromodeoxyuridine (BrdU; 20 mg/kg dissolved in 70 m*M* NaOH, 0.9% NaCl; Sigma) injections. Mice injected with NaOH/NaCl solution were used as negative control. These animals were sacrificed 1 day thereafter. Survival of labeled cells was checked in a group of mice that received BrdU 4 days after KA and were sacrificed after 1, 4, and 14 days.

Treatment with the antimitotic drug Azacytidine (15 mg/kg i.p.; Sigma Aldrich; Hossain et al., 1996; Nakayama et al., 1999), a drug that blocks DNA methylation, was performed to check the eventual recovery of dividing cells; BrdU incorporation was examined 1, 4, and 30 days thereafter. Azacytidine treatment did not produce signs of cell damage or glial reaction but effectively reduced the number of BrdU-positive cells. Finally, a group of animals received KA either 4 or 30 days after the antimitotic and BrdU injections were administered 4 days after KA. Azacytidine treatment did not modify either the susceptibility to KA or the pattern of seizures or the development of seizure-induced hippocampal lesions.

Adequate measures were taken to minimize pain or discomfort of animals (experiments were carried out in accordance with the European Communities Council, directive 86/609/EEC). Animals were perfused through the heart, under deep anesthesia with pentobarbital, with buffered 4% paraformaldehyde. After fixation, brains were kept 24 h in PBS containing 15% sucrose at 4°C. Labeling procedure was performed at room temperature on 40 μm vibratome coronal sections. Sections were first treated with 0.1% hydrogen peroxide (H_2O_2) in 1X PBS for 10 min, then in 1X PBS, 3% normal goat serum, 0.1% Triton X-100 for 60 min. Sections were then incubated 20 min at 45°C in 1 *M* HCl. They were then incubated overnight at 4°C with the primary antibody (monoclonal rat anti-BrdU, 1:50; Harlan SeraLab,

Loughborough, England), in blocking buffer (1X PBS, 1% BSA, 0.5% gelatin, 3% normal goat serum, 0.1% Triton X-100), and then finally incubated with goat antirat IgG/biotinylated (1:400; Vector Laboratories, Burlingame, CA) for 90 min followed by an avidin-biotin-peroxidase solution (Vectastain Elite ABC; Vector Laboratories); this later reaction was developed with 0.6 mg/mL diaminobenzidine (DAB; Sigma) in 0.05 *M* Tris, pH 7.4, 0.3% H_2O_2. Double labeling experiments to identify the phenotype of newly generated cells were performed by using rat-BrdU antibody revealed with antirat FITC (Jackson ImmunoResearch, West Grove, USA) and then one of the following mouse antibodies to: NeuN (1:1000; Chemicon, Temecula, USA), Nestin (1:1000; BD Pharmigen), Mash-1 (1:500; kindly provided by Dr Johnson), followed by goat antimouse Texas Red (1:200; Jackson ImmunoResearch) or rabbit anti-GFAP (1:200; Sigma Aldrich), followed by goat antirabbit-rhodamine. To check for the presence of apoptosis or cell damage, hippocampal sections from kainate-treated mice were stained with activated caspase-3 antibodies (1:5000; CM1, a gift of Tomaselli and Srinivasan) or TUNEL (In Situ Cell Death Detection Kit, Fluorescein; Boehringer) accordingly to the instructions of manufacturers. Brain sections from E17 mice were used as positive controls and sections incubated in the absence of antibodies or terminal transferase were used as negative controls.

Quantitative Analysis of BrdU-Labeled Cells

To evaluate the number of BrdU-positive cells, four nonadjacent coronal sections (40 μm thick) were selected from the midseptal part of the hippocampus, localized within −3.14 and −3.80 mm from bregma (Paxinos and Watson, 1998). To avoid labeling differences between hippocampal levels, particularly important for CA1 (Rietze et al., 2000), only this rostral part of the hippocampus was taken into account. The number of positive cells was counted in the hilar field for the DG and in the alveus, oriens, and pyramidal cell layers of CA1 using a 20X objective. DAB-revealed sections were analyzed with a computer-assisted stereological system (Neurolucida; Microbrightfield) with a Nikon microscope. Quantitative analysis of fluorescence preparations (BrdU+ colabeled by other antibodies, 40 μm thick sections) was realized on images recorded with a laser scanning confocal microscope (Olympus FV 300) and analyzed using the ImageJ 1.30h program (Java 1.3.1-03; Wayne Rasband, NIH, USA). The analysis of BrdU+NeuN+ cells was performed on serial optical sections (z-stack analysis). Only nuclei rating between 8 and 12 Java 1.3.1-03m diameter were taken into account.

Primary Culture and Cell Passaging

Hippocampal coronal sections (around 400 Java 1.3.1-03m thick) from adult CD1 mice were obtained and the CA1 (which also included the periventricular field) and DG (which also included the CA4 area) were dissected out with tungsten needles. The tissues were cut into smaller pieces (approximately 0.5 mm^3) and transferred into spinner flasks (Bellco Glass) with a magnetic stirrer filled with low Ca^{2+}, high Mg^{2+}, aCSF [containing 124 m*M* NaCl, 5 m*M* KCl, 3.2 m*M* $MgCl_2$, 0.1 m*M* $CaCl_2$, 26 m*M* $NaHCO_3$, 10 m*M* D-glucose, and penicillin-streptomycin 1:25 (pH 7.35, ≈280 mosmol)], and an enzyme mixture (1.33 mg/mL of trypsin, 0.67 mg/mL of hyaluronidase, and 0.2 mg/mL of kynurenic acid). The stirring tissue suspension was aerated with 95%O_2–5%CO_2 at 32 to 35°C for 60 min. Following this enzymatic incubation period the tissue was transferred to DMEM/F12 medium (1:1; Gibco BRL, Cergy Pontoise, France) containing 0.7 mg/mL of ovamucoid (Sigma) and triturated mechanically with a fired-narrowed Pasteur pipette. The dissociated cell suspension was centrifuged at 500×*g* for 5 min and the pellet washed once and then plated (15,000 viable cells/well) in noncoated 6-well Nunc tissue culture dishes. The growth media contained DMEM/F-12 nutrient (1:1), glucose (0.6%), glutamine (2 m*M*), sodium bicarbonate (3 m*M*), and HEPES buffer (5 m*M*) (all from Sigma except glutamine; GIBCO), insulin (25μg/mL), transferrin (100 μg/mL), progesterone (20 n*M*), putrescine (60 μ*M*), and selenium chloride (30 n*M*). EGF (Sigma; 20 ng/mL) and FGF-2 (RandD Systems; 20 ng/mL combined with 2 μg/mL heparin sulfate from Sigma), alone or combined were added to this media. The experiments were repeated eight times with three animals per experiment.

Renewal-Expansion Assay—Single Sphere Dissociation

Under sterile conditions single spheres of similar sizes (selected under a microscope equipped with a grid and ranging from 250 to 300 μm in diameter) were transferred to 500 μL Eppendorf tubes containing 200 μL of medium, mechanically dissociated as described before (Weiss et al., 1996a), and plated into a 96-well plate in the presence of the mitogens in which they were grown. The plates were scored 7 days later for the number of spheres derived from a single sphere. Only the spheres that were completely dissociated (no cellular clumps or sphere fragments observed the day following the dissociation) were considered.

Differentiation of Single Stem Cell-Generated Spheres

Seven days after the primary or secondary culture, spheres were removed with a pipette, spin down at 500×*g*, and resuspended in defined medium. The spheres were differentiated in single sphere cultures. Single isolated spheres were selected under a microscope equipped with a grid (ranging from 250 to 300 μm in diameter) and were plated on poly-*O*-ornithine-coated (15 μg/mL) glass coverslips in individual wells of 24- well Nunclon (1.0 mL/well) culture dishes. Because spheres generated from adult tissue differentiated in the presence of serum (FCS; 1%) do not generate neurons we used for the differentiation assay the same protocol as described before (Represa et al., 2001). In brief,

spheres were incubated for 5 days in the mitogen(s) with which they were generated, followed by 5 days in DMEM/F-12 medium with the hormone and salt mixture but no mitogens. Medium was not changed for the rest of the experiment. Coverslips were processed for indirect immunocytochemistry.

Immunocytochemistry

Triple indirect immunocytochemistry of these spheres was performed as described before (Reynolds and Weiss, 1996; Weiss et al., 1996a) to check the capacity to generate the three main cell types. The antibodies used included: mouse monoclonal antibody (IgG) to MAP-2 (1:1000; Boehringer Mannheim, Meylan, France), rabbit polyclonal antibody to GFAP (1:400; Sigma), and mouse monoclonal antibody (IgM) to O4 (1:20; Boehringer). Secondary antibodies (all from Jackson ImmunoResearch) were: rhodamine-conjugated affinity-purified goat antimouse IgG (1:200), AMCA-conjugated affinity-purified goat antirabbit IgG (1:100), fluorescein-conjugated affinity-purified goat antimouse IgM (1:100). Nonimmune immunoglobulins instead of primary antibodies were used as controls for the specificity of stainings.

Organotypic Slice Culture

Organotypic slices were prepared from hippocampi of P10 male Wistar rats according to the procedure described by Stoppini et al. (1991) with minor changes. Briefly, rats were decapitated under deep anesthesia with chloral hydrate (i.p., 350 mg/kg). Following the removal of the brain, hippocampal sections, 400 μm thick, were prepared using a chopper (Mc Ilwain, U.S.A.) and collected in a PBS solution supplemented with 0.5% D-glucose. Slices were then kept at 4°C for 30 min in PBS-glucose and then placed onto Millicell-CM culture inserts (0.4 μm; Millipore). The inserts were placed into 6-well culture plates with 1 mL MEM (Sigma) supplemented with: glucose (25 m*M*), HEPES (30 m*M*), $Na_2CO_3.H_2O$ (5 m*M*), L-glutamine (3 m*M*), L-ascorbic acid (0.5 m*M*), $CaCl_2$ (2 m*M*), $MgSO_4$ (2.5 m*M*), insulin 1 mg/L, and phenol red (2 mL/L of 0.5% solution), pH 7.25–7.29, and horse serum (20%; Sigma). Slices were maintained for 2 weeks in a cell culture incubator at 37°C, in an atmosphere containing 5% $C0_2$. The medium was changed three times per week. At 15 days *in vitro* (DIV), growth factors EGF or FGF-2 were added directly to the culture medium (20 ng/mL every 12 h for 48 h). Forty-eight hours after the first application of growth factors two successive pulses of BrdU (1 μM, every 2 h) were applied and cultures fixed 2 h later by immersion for 1 h at room temperature in 4% paraformaldehyde. BrdU and Nestin (1/500; Pharmingen) immunostainings were performed as described before. Experiments were repeated five times.

Statistics

Significant differences were checked using ANOVA followed by Mann-Whitney *U* test to compare mean values from different culture experiments and animals. Values expressed as percentage (e.g., number of neurons) were analyzed using x^2-test.

RESULTS

Precursor Cells in the DG and CA1 Fields

To investigate the presence of precursor cells we analyzed the distribution of BrdU-labeled cells on sections from adult mice sacrificed 12 h after the last BrdU injection (Fig. 1) combined with the detection of either one of the two following markers of neural progenitors: Nestin, an intermediate filament mainly expressed by stem cells (Lendahl et al., 1990), though it may also be expressed by more mature cells (Zerlin et al., 1995), or Mash-1 (mammalian achaete-scute homologue 1; Johnson et al., 1990; Lo et al., 1991). Mash 1 is also expressed in developing hippocampus and it has been shown that the number of Mash-1-positive precursors rapidly increases in the dentate gyrus and CA1 field of ischemic animals or after the treatment with EGF+FGF-2 (Nakatomi et al., 2002). In the DG, BrdU-labeled cells localized mainly in the subgranular zone [Fig. 1(a)]. Cells labeled with Nestin were also observed in this layer [Fig. 1(c)]; Nestin-immunoreactivity decorated cell bodies and in some cases radial-like extensions. Cells BrdU+ and Nestin+ were observed in the subgranular zone [around 20% of BrdU cells; Fig. 1(e)]. Mash-1 staining in the DG was close to background (rather nonspecific) and no clear colocalization with BrdU was observed. In CA1, BrdU-positive cells were also observed [Fig. 1(b)]. They localized in the subventricular zone but were also present in the oriens, pyramidal, and radiatum strata as reported before (Rietze et al., 2000; Nakatomi et al., 2002). Nestin- [Fig. 1(d,f)] and Mash-1- [Fig. 1(g–j)] positive cells were also present in these layers. While Nestin clearly stained soma and extensions, Mash-1 staining was restricted to the nuclear compartment as expected. BrdU-positive cells also coexpressed these precursor cell markers [Fig. 1(e–j)].

To investigate the respective capacities of DG and CA1 to generate neurons and glial cells, animals sacrificed 7 days after the last BrdU injection were stained with BrdU and glial (GFAP) or neuronal (NeuN) markers: double labeled cells were observed

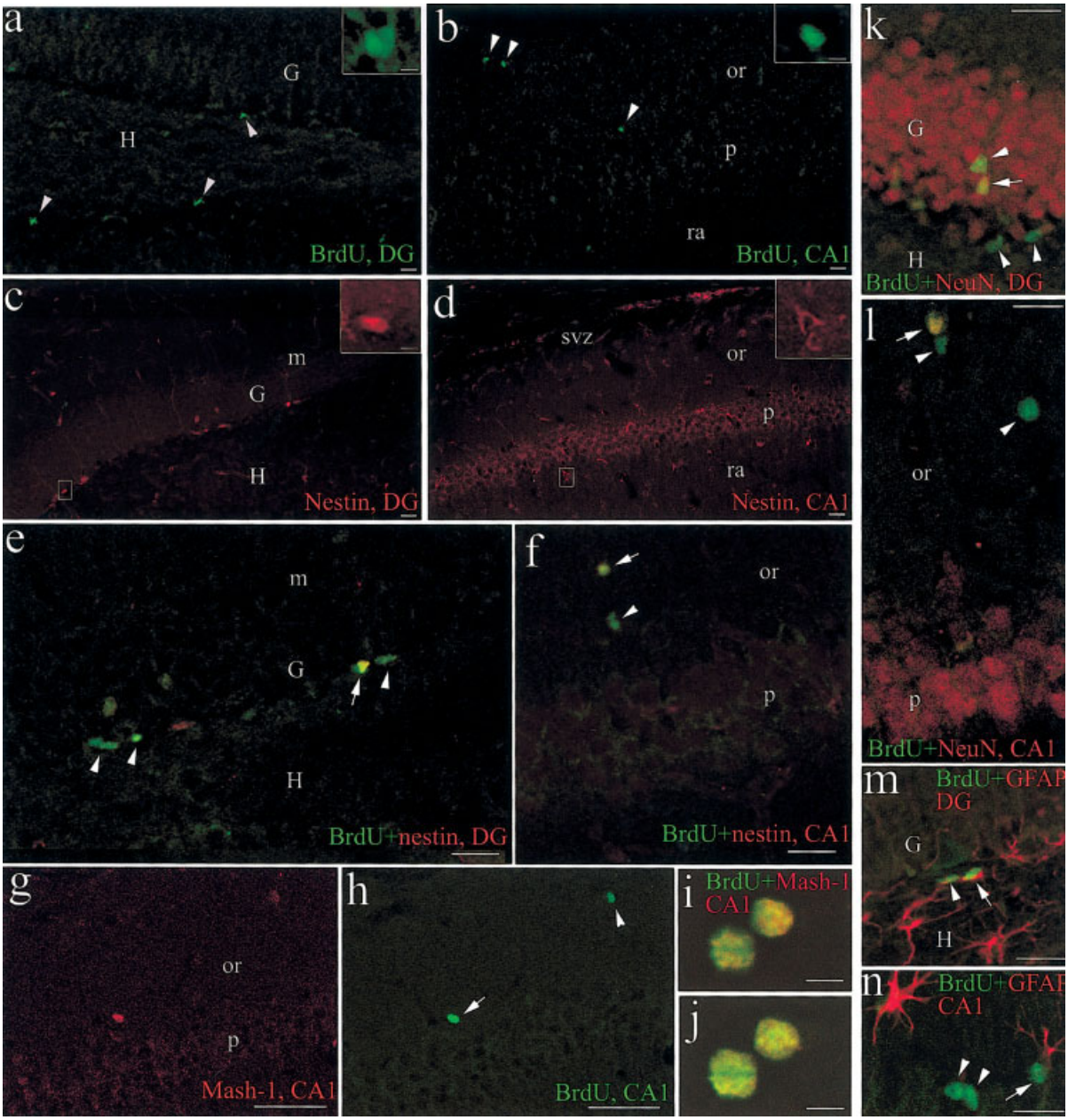


Figure 1 Neural progenitors in adult mouse hippocampus. BrdU staining (revealed in green) depicts the presence of proliferating cells in the DG (a,e,k,m) and CA1 field (b,f,h–j,l,n) of adult mice. Some BrdU-positive cells were observed to be immunoreactive to (arrows) Nestin (e,f) or Mash-1 (g–j) in mice 12 h after the last BrdU injection, or NeuN (k,l) and GFAP (m,n) in mice sacrificed 7 days later. (a) BrdU staining of DG. Inset depicts at a higher magnification two labeled cells. (b) BrdU staining of CA1. Inset depicts at a higher magnification one labeled cell. (c) Nestin immunostaining of DG. Insets in (a) and (b) show typical BrdU labeled cells. Insets in (c) and (d) represent the enlargement of the field in the rectangle; (d) Nestin immunostaining of CA1; (e) double BrdU plus Nestin (in red) immunostaining of DG; (f) BrdU plus Nestin (in red) immunostaining of CA1; (g) and (h) BrdU [in (h)] plus Mash-1 [in (g)] immunostaining of CA1; (i) and (j) two different confocal planes of BrdU+Mash-1-positive cells in the stratum oriens of CA1; (k) and (l) BrdU+NeuN immunostaining of DG (k) and CA1 (l). (m) and (n) BrdU+GFAP immunostaining of DG (m) and CA1 (n). Arrows depict double labeled cells. Arrowheads depict cells positive to BrdU. Abbreviations: G, granule cell layer; H, hilus; m, molecular layer; or, stratum oriens; p, stratum pyramidale; ra, stratum radiatum. Scale bar = 50 μm (a–h), 10 μm (i,j), 20 μm (k–n), 5 μm in (a–d) insets.

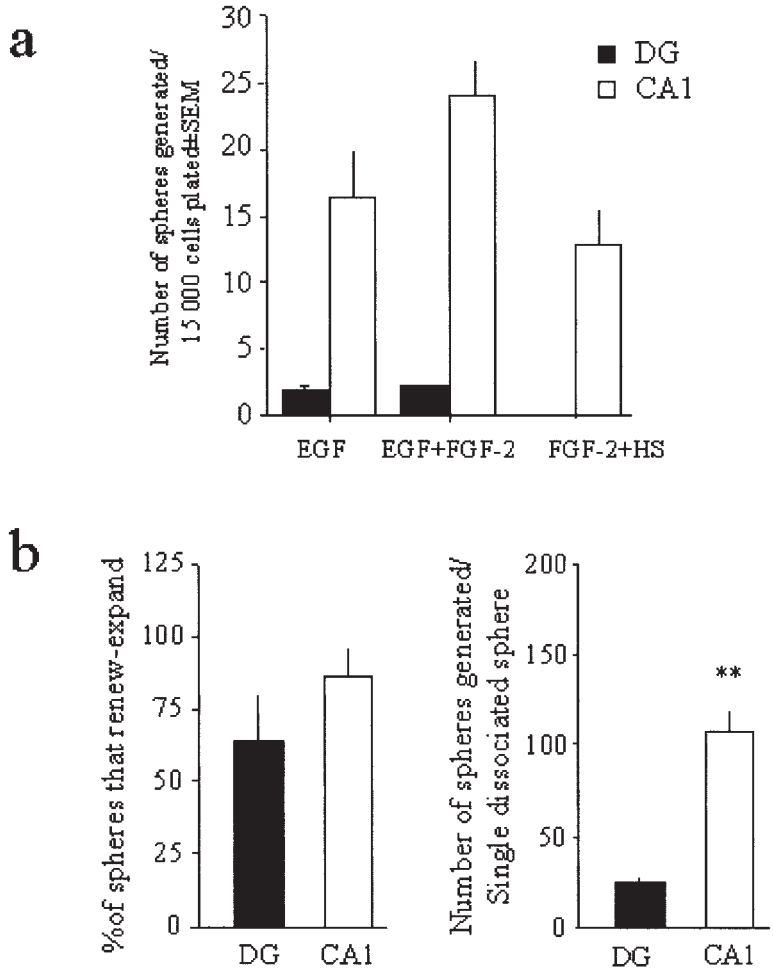


Figure 2 Neurosphere-forming cells isolated from adult CA1 displayed different trophic factor responses and expansion properties than dentate gyrus cells. (a) Number of neurospheres generated by adult hippocampal precursor cells. Cells isolated from DG and CA1 field were cultured (15,000 cells/single 35 mm well) in the presence of either EGF (20 ng/mL) or FGF-2 (20 ng/mL, plus 2 μg/mL heparan sulfate) or both, and the numbers of spheres generated were analyzed after 7 DIV. Dentate gyrus cells, unlike CA1 cells, did not form spheres when cultured in the presence of FGF-2 (+heparan sulfate) alone. (b) Expansion-renewal of adult hippocampal stem cells isolated from DG and CA1. Single neurospheres (around 300 μm diameter) grown in EGF were isolated and mechanically dissociated in normal culture media. They were subcloned in the presence of EGF and the number of newly generated spheres was rated after 7 DIV. Dentate gyrus neurospheres produced less secondary clones than CA1 neurospheres (**$p < 0.001$).

in both fields [Fig. 1(k–n)]. In the DG, BrdU+ GFAP+ cells were observed within the subgranular zone; the nuclei of these cells were rather oblong [Fig. 1(m)]. BrdU+NeuN+ cells distributed mainly within the granule cell layer. In CA1, BrdU+ GFAP+ were observed in all layers, including the stratum oriens, and their nuclei were rather round [Fig. 1(n)]. BrdU+NeuN+ cells were observed in the stratum oriens and eventually the pyramidal cell layer or the stratum radiatum as described before (Rietze et al., 2000). Taken together, these data suggest, in agreement with previous observations (see Introduction), the presence of multipotent precursors in both CA1 and DG.

Neurosphere-Forming Cells from the Adult CA1 and DG Regions of the Hippocampus Display Different Properties

In order to explore the cellular characteristics of proliferation and self-renewal in the CA1 and DG re-

Table 1 Multipotent Hippocampal Precursors Are Mainly Localized in CA1

	Number of Clones	N+A+O	N+A	A+O/O
Dentate gyrus				
EGF	50	5	4	41
FGF-2 (+HS)	—	—	—	—
CA1				
EGF	26	25	0	1
FGF-2 (+HS)	43	41	0	2

Single-cell-generated neurospheres grown in the presence of EGF or FGF-2 were plated on poly-L- ornithine coated coverslips in normal media containing 1% FBS for 7 days. Triple labeling immunostaining was performed after aldehyde fixation with antibodies specific for neurons (MAP-2), astrocytes (GFAP), and oligodendrocytes (O4) as indicated in Methods. The majority of CA1 neurospheres were multipotent (N+A+O) while DG neurospheres displayed a more limited capacity to generate neurons (N) and mainly generated astrocytes (A) and oligodendrocytes (O).

gions, we examined the properties of neurosphere-forming cells (presumptive neural stem cells) derived from each region. It has been previously shown that neural stem cells form neurospheres, which contain various proportions of neurons and glia, and when dissociated have the capacity to generate secondary neurospheres (Gritti et al., 1996). Equal numbers of dissociated cells from the CA1 and DG were cultured in the presence of either EGF (20 ng/mL), FGF-2 (20 ng/mL + 2 μg/mL of heparin sulfate), or EGF+FGF-2 combined. After 7 DIV, the absolute numbers of neurospheres were counted (Fig. 2). Whether cultured in EGF alone or with EGF+FGF-2, fewer than five neurospheres were generated from 15,000 starting cells derived from the DG. Absolutely none were generated in the presence of FGF-2 alone. At the same time, all three conditions supported the generation of neurospheres from the CA1 regions, generating between 10–25 neurospheres from 15,000 starting cells. These results suggest that the CA1 region is enriched in neurosphere-forming cells, which can respond to either mitogen. On the other hand, the DG contains fewer neurosphere-forming cells and none of them responded to FGF-2 alone.

We then compared the fate properties of neurosphere-forming cells from the adult CA1 and DG. Single-cell-generated spheres were plated on poly-*O*-ornithine coverslips, cultured for 10 DIV, and subsequently analyzed for the presence of neurons, astrocytes, and oligodendrocytes (Table 1). Virtually all neurospheres generated from CA1, whether derived with EGF (25/26) or FGF-2 (41/43), contained neurons, astrocytes, and oligodendrocytes. Neurospheres generated from the DG in the presence of EGF contained mainly glial cells (41/50) and no more than 10% contained the three cell types. These results suggest that neurosphere-forming cells from the DG are restricted in the phenotypes they produce, while those derived from the CA1 are virtually all multipotent.

We then compared the renewal/expansion properties of CA1 and DG EGF-generated neurospheres of equal size [Fig. 2(b)]. While the frequency of self-renewal (ability of an EGF-generated neurosphere to produce more than one of itself) was not different between CA1 and the DG, the absolute numbers of secondary neurospheres was [Fig. 2(b)]. CA1 EGF neurospheres produced four times more secondary neurospheres than those derived from the DG [Fig. 2(b)]. These findings suggest that EGF-generated neurospheres derived from the DG exhibit fewer expansion properties than those derived from the CA1 region.

EGF and FGF-2 Differentially Modulate the Proliferation of CA1 and DG Precursors on Organotypic Explants

It has been previously suggested that FGF-2 effects on cell proliferation may require the presence of endogenous factors released by adjacent cells; these factors would be absent from low-density cultures. In addition, dissociated cells may represent a mix of cells from different layers, and, in the case of the CA1 field, they may involve precursors localized in the periventricular zone and/or precursors localized in the remaining layers (also see Seaberg et al., 2002). We then explored the effects of EGF and FGF-2 on organotypic hippocampal explants, a procedure that allows the maturation of the hippocampus *in vitro* (in this study at 15 DIV), while maintaining the same cytoarchitectonic organization and cell diversity as *in vivo* (Stoppini et al., 1991). Slices were treated four times with FGF-2 or EGF during 48 h and then fixed. We observed that EGF treatment enhanced the cell proliferation in the CA1 but not in the DG [Fig. 3(b,e)]. In CA1, the majority of BrdU-positive cells were localized in the stratum oriens [Fig. 3(e)]. A few labeled cells were also observed within the

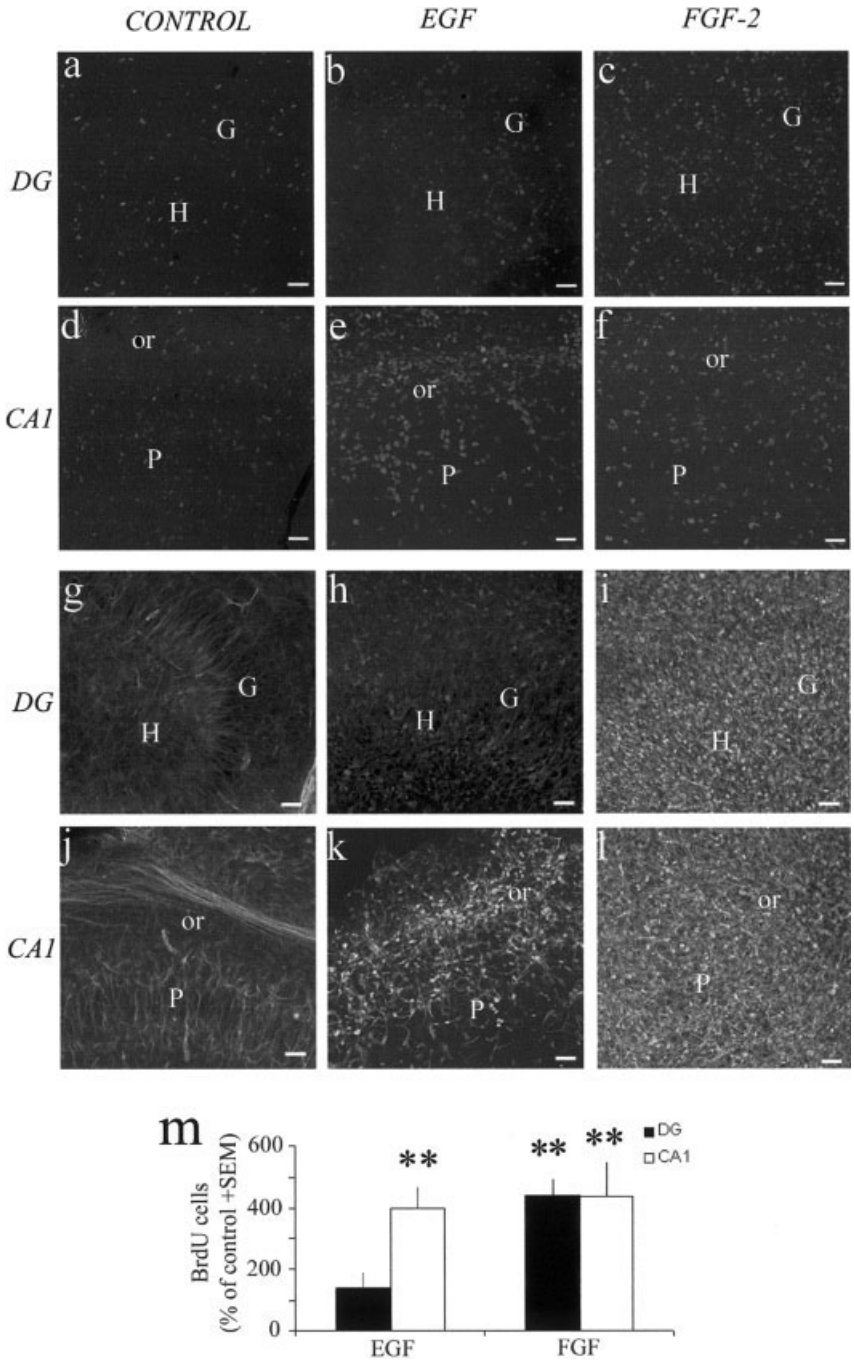


Figure 3 BrdU incorporation and Nestin immunoreactivity in organotypic hippocampal explants treated with EFG or FGF-2. Mature organotypic hippocampal explants (15 DIV) were treated for 48 h with either EGF (b,e,h,k) or FGF-2 (c,f,i,l) and compared to control, untreated slices (a,d,g,j). After a subsequent incubation with BrdU for 24 h, sections were fixed and stained with BrdU (a–f) or Nestin (g–l). Note that EGF produced a quite selective effect on CA1. In contrast, FGF-2 treatment induced a widespread effect not field or layer specific. The number of BrdU-positive cells was rated and expressed in (m) as percentage of positive cells in control explants (mean data from 21 to 27 organotypic slices/condition from five independent experiments; $^{**}p < 0.001$ as compared to control).

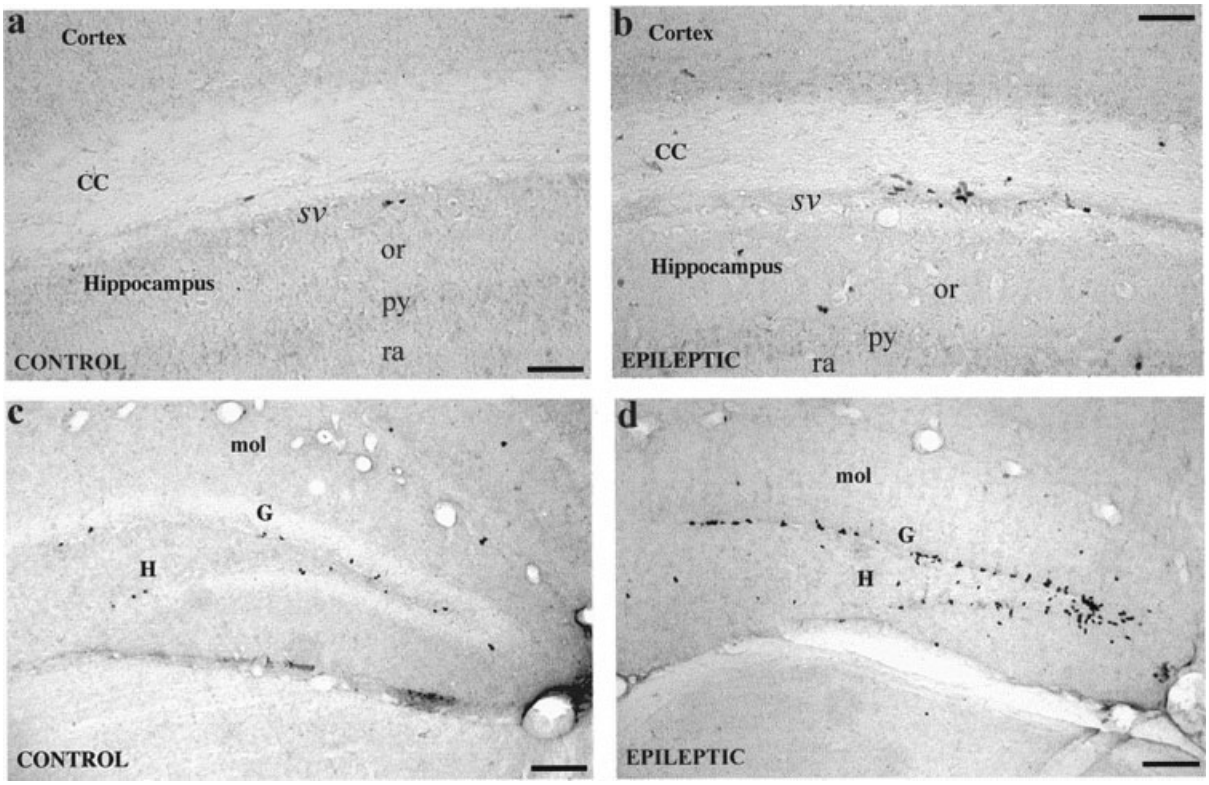


Figure 4 BrdU incorporation in control and epileptic adult hippocampus. BrdU staining was obtained on hippocampal sections from control or epileptic mice. BrdU was injected 3 days after treatment with the convulsant agent kainic acid and the animal was sacrificed 24 h later. Labeled cells may be observed in the stratum oriens and subventricular area (CA1; upper pictures) and the subgranular layer of the DG (lower pictures). CC, corpus callossum; sv, subventricular. Scale bars = 100 μm.

strata pyramidale and radiatum and the subventricular zone. The treatment with FGF-2 enhanced the cell proliferation on the entire slice and did not reveal a specific site of action [Fig. 3(c,f)]. The analysis of Nestin immunoreactivity depicted similar features [Fig. 3(g–l)]: EGF dramatically increased the immunoreactivity in CA1 [Fig. 3(k)] but not in the DG [Fig. 3(h)], and most of the Nestin- immunoreactive cells localized within the stratum oriens. The treatment with FGF-2 produced a widespread effect and increased Nestin immunoreactivity in a nonspecific site form [Fig. 3(e,i)]. Altogether, these data support EGF as a quite selective trophic factor for CA1 precursors and suggest that FGF-2 acts as a noncell specific mitogenic factor.

In Vivo "Expansion" of CA1 and DG Precursor Cells

In previous studies, seizures were shown to induce a dramatic increase in proliferation and generation of new granule cells in the DG of the adult hippocampus (Bengzon et al., 1997; Parent et al., 1997). Consequently, seizures may be a good tool to investigate the expansion capacities of hippocampal precursors *in vivo*. Thus, we asked whether the convulsant agent KA could enhance proliferation in the CA1 region of the adult hippocampus, and, if so, how this compares to that occurring in the DG. We first examined the pattern of BrdU incorporation in the hippocampus, 4 days after KA-induced seizures (BrdU was injected 24 h before sacrifice). In untreated (control) animals, BrdU staining was detected in both CA1 [Fig. 4(a)] and DG [Fig. 4(c)]. The staining in CA1 was concerned in the stratum oriens and the subventricular zone; only a few BrdU-positive cells were observed in the pyramidal cell layer and stratum radiatum. Kainate treatment resulted in an enhanced proliferation in both the dentate subgranular zone [Fig. 4(d)] and CA1 [Fig. 4(b)]; in this latter field, BrdU-positive cells localized in both the subventricular zone and the stratum oriens (Fig. 4). These results suggest that

proliferation in distinct regions of the hippocampus may be modulated by seizures. Alternatively, BrdU may label in CA1 cells repairing their DNA after insult (Yang et al., 2001). In fact, KA treatment induced a dramatic cell death of hippocampal neurons that occurred during the first 3 days following the treatment (Represa et al., 1995). This damage might involve both pyramidal cells and interneurons. In the present study we used Cresyl violet to investigate for cell pyknosis and TUNEL and activated caspase-3 stainings to check for the presence of damaged neurons in CA1 and compare these results with BrdU staining.

Signs of CA1 pyramidal cell damage were observed in only 3 out of 19 animals sacrificed 3 days after KA, this including pyramidal cell layer disruption, cell pyknosis, and glial hypertrophy (increase of GFAP staining; not shown). In addition, cell degeneration was also observed in the stratum oriens but only of cells close to the pyramidal cell layer. All animals investigated presented a clear increase in the number of BrdU-positive cells in the stratum oriens and subventricular area, independent of the presence or absence [Fig. 5(c)] of cell damage. The CA1 field of animals sacrificed 15 days after KA was essentially normal (six out of seven); only one animal displayed a loss of neurons in the pyramidal cell layer and stratum oriens and a prominent glial reaction (one out of seven). No signs of cell pyknosis or swelling were detected by this period. The increase of BrdU staining was observed in the whole population of mice. Animals sacrificed 30 days after KA were either normal (four out of five) or presented CA1 cell loss (one out of five). For subsequent quantitative analysis only animals with no apparent lesions in CA1 were considered.

In mice sacrificed 3 days after KA or thereafter, activated caspase-3 immunopositive cells were never observed in CA1 [Fig. 5(g)], and only a few isolated cells could be eventually detected in the cortex, dentate gyrus [Fig. 5(h)], and amygdala. Similarly, TUNEL-positive cells were not observed in CA1 [Fig. 5(d)] and only a few positive cells and debris were detected in CA3 and cortex [Fig. 5(e,f)]. Cortical sections from E17 mice used for positive control displayed the presence of TUNEL [Fig. 5(i)] and activated caspase-3-positive [Fig. 5(j)] cells. Taken together these observations suggest that BrdU labeling in CA1 is independent of cell apoptosis or DNA damage and repair.

To further confirm that BrdU-positive cells were newly generated cells and not damaged-degenerating cells we investigated the pattern of BrdU incorporation in animals injected with BrdU at different times after KA and sacrificed 12 h later [Fig. 6(a)] or 4 days after KA and sacrificed 12 h, 7, or 15 days thereafter [Fig. 6(b)]. The number of BrdU+ cells was increased in both CA1 and DG between 3 and 15 days after KA and returned to control values 30 days after KA. The duration of the increased proliferation was therefore longer than the window of cell death, which was almost completed by the third day following seizures. Interestingly, the increase was more pronounced in CA1 than in dentate gyrus, suggesting a higher expansion of precursors in the former region. The analysis of stained cells at different times after BrdU injection demonstrated that BrdU+ cells persisted in both regions during at least 2 weeks, indicating that labeled cells were not dying cells. In addition, only a few cells were positive in the pyramidale and radiatum strata. In the dentate gyrus, however, the number of BrdU+ cells decreased in mice sacrificed 15 days after BrdU as compared to animals sacrificed at shorter delays [Fig. 6(b)], indicating a worse survival of BrdU+ cells in DG than in CA1. In fact, it has been previously shown that a large proportion of cells that proliferate in the adult dentate gyrus under normal conditions or in response to brain insults exhibit only short-term survival (Ekdahl et al., 2002). Taken together, these data strongly support the notion that in CA1, 4 days after KA, dying pyramidal neurons did not incorporate BrdU and that BrdU- labeled cells were not damaged-degenerating neurons. Therefore, the present described increased BrdU staining in the stratum oriens and subventricular zone likely represents a staining of dividing cells.

To further confirm that seizures resulted in the expansion of CA1 and DG precursors 4 days after KA, animals were stained with BrdU and Nestin or Mash-1 antibodies (Fig. 7). In the DG Nestin immunoreactivity was enhanced after KA [Fig. 7(b)] as compared to control mice [Fig. 7(a)]. This antibody in this field decorated cell bodies localized in the subgranular zone as well as many "radial-like" extensions. These immunopositive cells were eventually BrdU+. The number of double-stained cells was not estimated here because of the difficulty in identifying, in many cases, whether radial-like extensions emerged effectively from BrdU-positive nuclei or not. In CA1 Nestin immunoreactivity was dramatically increased in the oriens, pyramidale, and radiatum strata [Fig. 7(d)]. Up to 90% of BrdU-positive cells in this zone were Nestin positive. Nestin staining in CA1 differed from that in DG as immunoreactivity mainly concentrated in cell bodies and cells displayed only short thin extensions [Fig. 7(d) and inserts 1 and 2]. Mash-1 immunoreactivity clearly increased 4 days after KA in the DG and CA1 [Fig. 7(e–h)]. Mash-1 immunoreactivity in the DG localized mainly in the

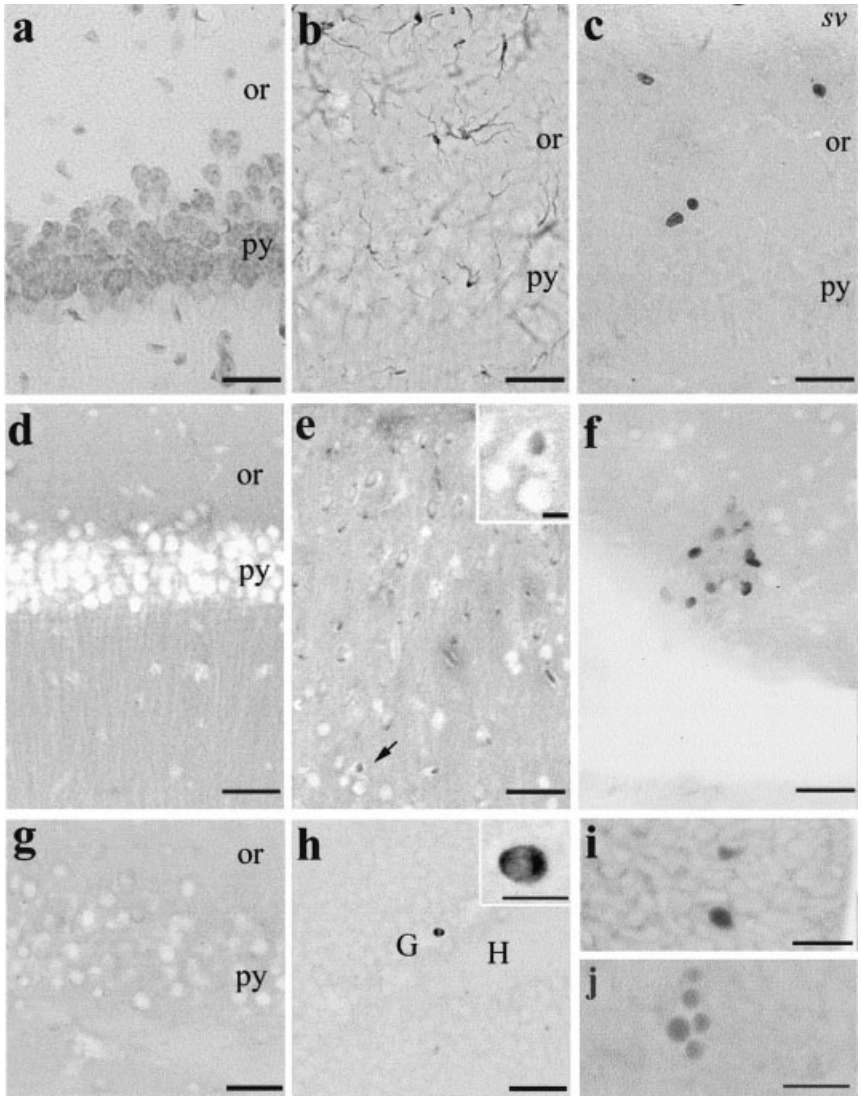


Figure 5 BrdU incorporation in CA1 reveals proliferating cells rather than damaged or degenerating neurons. (a–c) To confirm that BrdU stains proliferating cells in CA1, adjacent sections from mice sacrificed 3 or 4 days after KA were stained with either Cresyl violet (a) or GFAP (to stain astrocytes) (b) or BrdU (injected 24 h before) (c). No major signs of cell lesion or glial reaction were observed in 16 out of 19 animals as in that depicted in (a,b). In this animal, several BrdU-positive cells were observed in the stratum oriens (c). (d–f) TUNEL staining of brain sections from a mouse sacrificed 3 days after KA. The CA1 field (d) was devoid of any positive staining while TUNEL-positive nuclei and cellular debris in the somatosensory cortex [(e); insert is the enlargement of the cell pointed at by the arrow] and the retrosplenial cortex (f) were observed in the same animal. (g,h) Activated caspase-3 immunostaining of hippocampal sections from a mouse sacrificed 4 days after kainate. The CA1 field (g) was devoid of any positive staining while an isolated immunoreactive cell was detected in the dentate gyrus granule cell layer [(h); inset illustrates this positive cell at a higher magnification]. Similar results were obtained in seven different mice. TUNEL- (i) and caspase-positive (j) cells were observed in the cortex of E17 mice, used as positive controls. These observations reveal that BrdU incorporation in CA1 does not correlate with DNA damage or repair. Scale bars = 50 μm in (a–h), 20 μm in (i,j), and 10 μm in insets.

subgranular field, where it concerned 20 to 40% of BrdU-positive cells. Mash-1 staining in CA1 was mainly observed in the stratum oriens and subventricular zone and virtually concerned all BrdU-positive cells.

We next characterized the pattern of neuronogen-

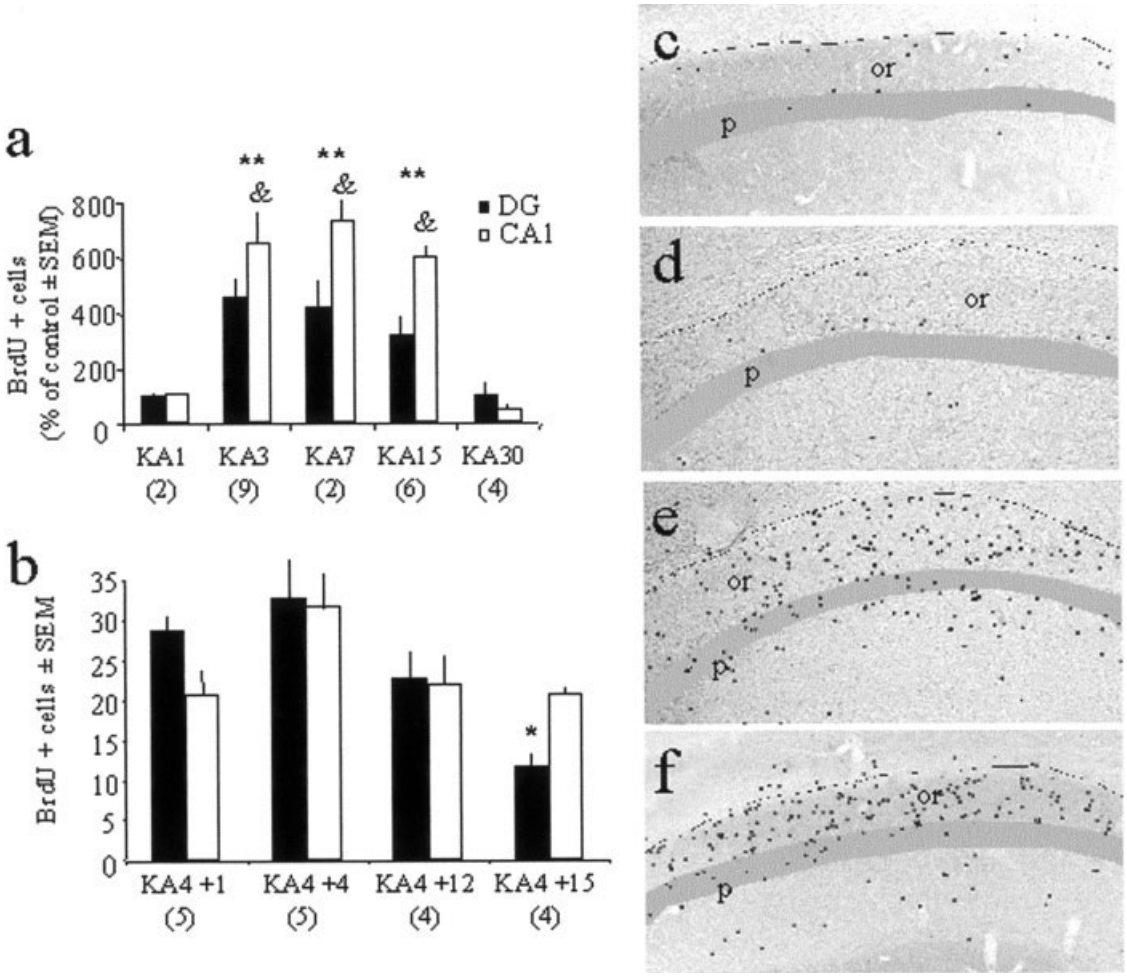


Figure 6 Cell proliferation in the DG and CA1 fields of control and epileptic mice. (a) Animals received six injections of BrdU at different times after the convulsant or vehicle (from 1 to 30 days) and the number of BrdU+ cells was rated 24 h thereafter in both the CA1 field (alveus, oriens, and pyramidal cell layers) and the DG (subgranular-granular cell layers). Increased proliferation was observed 3 days after KA but not before. This increased proliferation lasted for 15 days and returned to control values by 30 days after the injection. (b) Animals received six injections of BrdU 4 days after KA and were sacrificed 1 to 15 days later. The number of BrdU+ cells was relatively stable in CA1 (mean numbers ± SEM/mm^2) for the period of survival analyzed but decreased significantly in DG (mean number ± SEM/0.25 mm^2) during the second survival week (the number of mice analyzed per group in brackets). $^{**}p < 0.001$ as compared to control; $\&p < 0.05$ as compared to DG. (c–f) Camera lucida drawings to depict the distribution of BrdU-positive cells. Each draft represents the superposition of one section/animal from four mice per group. The borders of layers are outlined in gray. (c) Control mice sacrificed 12 h after the last BrdU injection. Cells were distributed within the stratum oriens and the subventricular area; a few positive cells were also observed in the pyramidale-radiatum strata. (d) Control mice injected with BrdU 15 days before. The distribution of BrdU-positive cells was similar to that in (c). (e) In mice injected with BrdU 3 days after KA and sacrificed 12 h after the last BrdU injection, numerous positive cells were detected. They were localized mainly in the stratum oriens and the subventricular area. Only a few cells were positive in the pyramidale-radiatum strata. (f) In mice injected with BrdU 3 days after KA and sacrificed 15 days thereafter a high number of positive cells was still detected; they distributed through the whole stratum oriens and subventricular area. These observations suggest that cells labeled by BrdU 4 days after KA do not degenerate and do not apparently migrate.

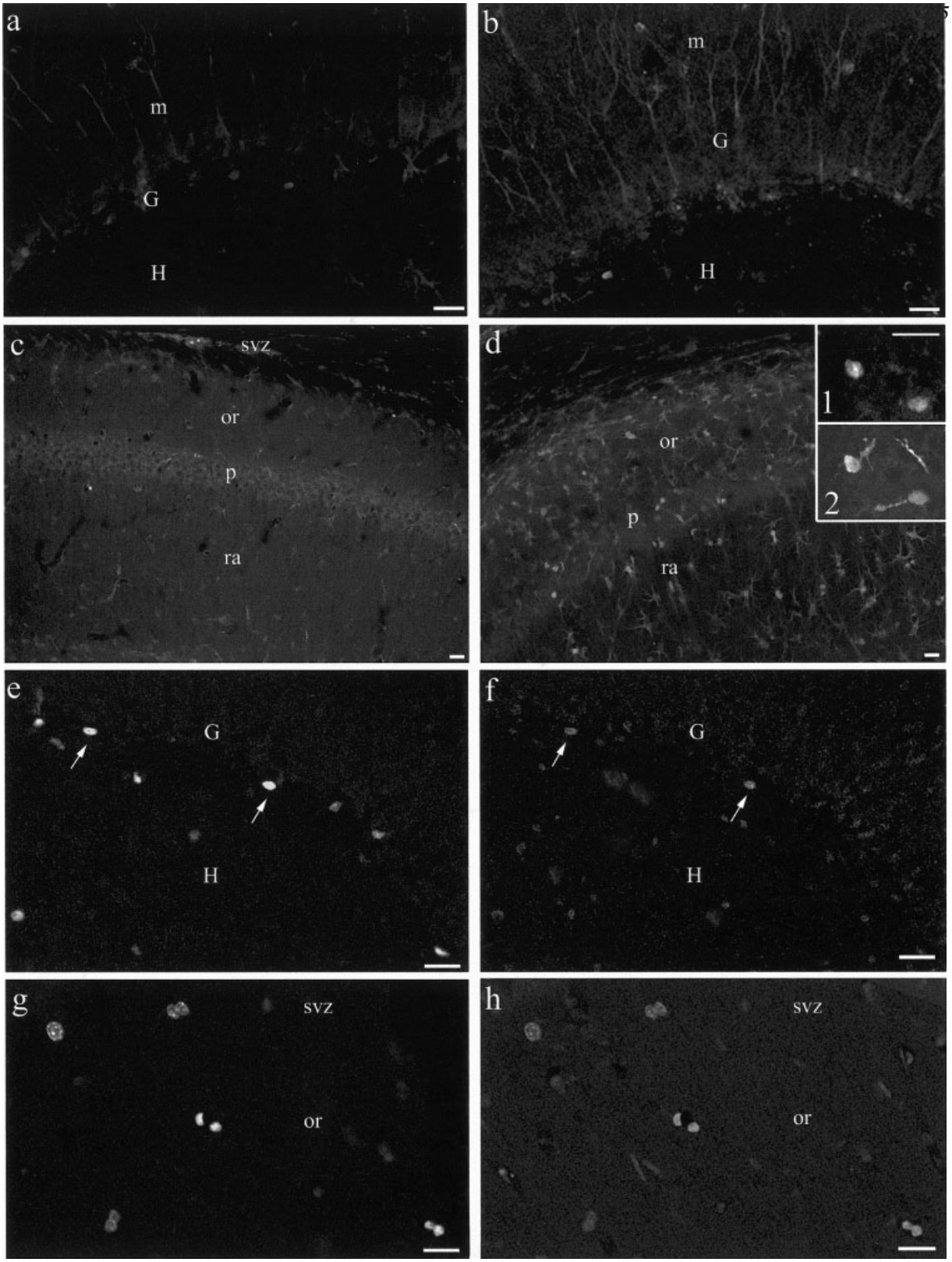


Figure 7 Precursor cells after kainate treatment. (a) Nestin immunoreactivity in the DG of control mice. (b) Nestin immunoreactivity in the DG of KA4 mice; note the radial-like staining through the granule cell layer. (c) Nestin immunostaining of control CA1. (d) Nestin immunostaining of KA4 CA1; immunopositive cells distribute though the oriens, pyramidal, and radiatum strata. Inserts depict double labeling with BrdU (1) and Nestin (2) in the stratum oriens. (e,f) Double staining of KA DG with BrdU (e) and Mash-1 (f). (g,h) Double staining of KA CA1 with BrdU (g) and Mash-1 (h). Arrows depict double stained cells. Scale bars = 20 μm.

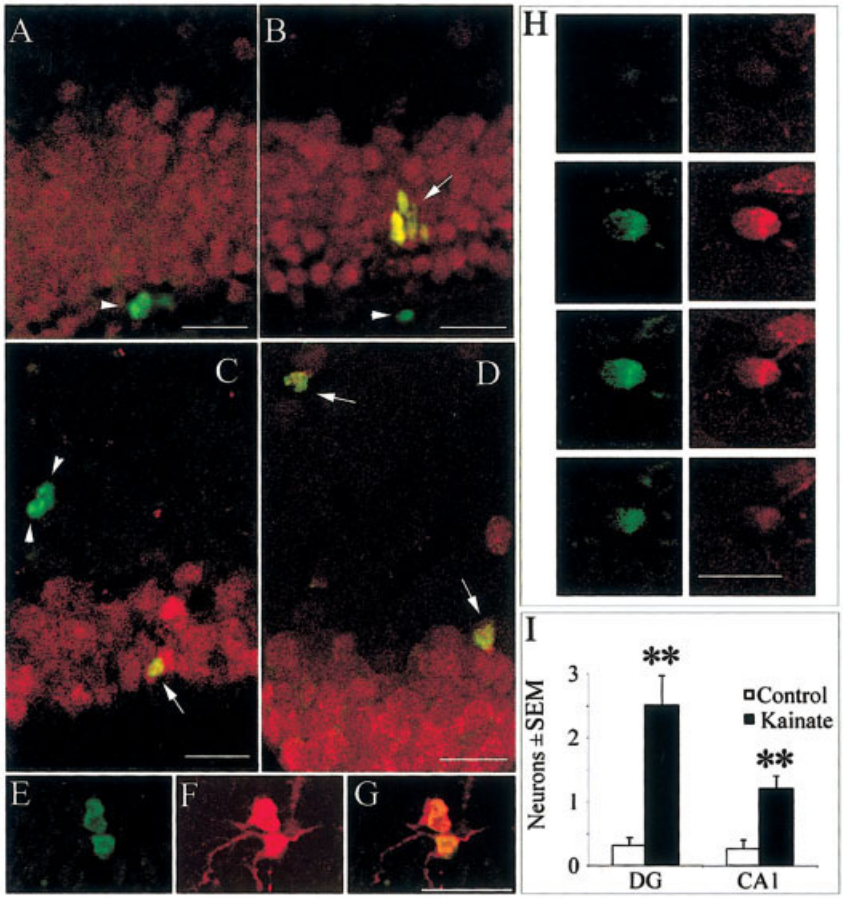
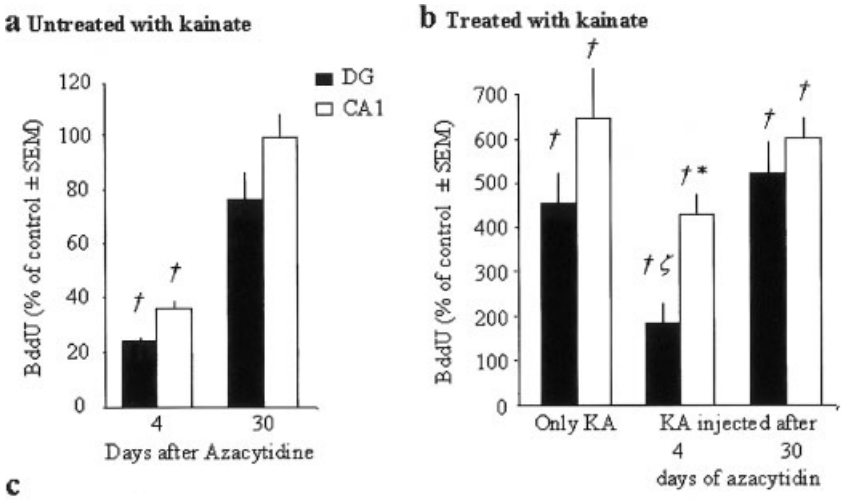


Figure 8 Genesis of new neurons increased in the hippocampus of epileptic mice. (A–D) Double immunostaining of DG (A,B) and CA1 (C,D) sections from control (A–C) or epileptic mice [injection with BrdU 4 days after KA; animals were sacrificed 7 days thereafter (B,D)]. BrdU was revealed in green and NeuN was revealed in red. (E–G) Double immunostaining of a CA1 section from an epileptic mouse (same as before) with BrdU (green) and calbindin (red) antibodies. These neurons localized within the stratum oriens and might represent interneurons. (H) Serial optical sections of cells double stained with BrdU (red) and NeuN (green) in the stratum oriens from a KA-treated mouse. Sequential pairs of pictures were taken every 2 μm; this confirms that the same cell nuclei stains for both antibodies. (I) Histograms depicting the number of BrdU+ neurons $\pm$ SE/mm^2 detected in the dentate gyrus and the stratum oriens of control ($n = 4$) and epileptic mice ($n = 4$ mice injected with BrdU 4 days after KA and sacrificed 7 days thereafter; $^{**}p < 0.001$ as compared to control). Scale bar = 20 μm. Arrowheads depict cells BrdU+NeuN−; arrows depict cells BrdU+NeuN+.

esis by a dual labeling of hippocampal sections from animals injected with BrdU 4 days after KA and sacrificed 7 days later ($n = 4$ mice). Double staining with NeuN (and in a few cases calbindin) was confirmed using a confocal microscope and doing serial optical sections (z-stack analysis) in a sequential way [Fig. 8(H)]. In CA1, new neurons (cells BrdU+ NeuN+) were principally found in the stratum oriens [Fig. 8(D)] and barely in the pyramidal cell layer [Fig. 8(C)] and radiatum. In the DG, double-stained cells were essentially observed within the granule cell layer [Fig. 8(B)]. Quantitative analysis confirmed a dramatic increase of BrdU+NeuN+ cells in both the CA1 and DG regions [Fig. 8(I)]. In CA1, BrdU cells positive to calbindin were also observed in the stratum oriens [Fig. 8(E–G)]. These cells were of small size and displayed short dendrites.

To further analyze the intrinsic expansion properties of CA1 and DG precursors *in vivo* we studied the renewal of cell proliferation, in both the CA1 and DG, following the administration of the antimitotic DNA methylating agent 5-azacytidine [Fig. 9(a)]. Twenty-four hours after 5-azacytidine administration (15 mg/kg i.p.), the numbers of BrdU-labeled cells in both the



c

Time (days)	*0*	*3*	*4*	*7*	*8*	*29*	*30*	*33*	*34*
Control (6)	Saline	BrdU	S						
KA only (9)	KA	BrdU	S						
KA 4d after Aza (5)	Aza		KA	BrdU	S				
Ka 30d after Aza (3)	Aza						KA	BrdU	S
Aza 4d (4)	Aza	BrdU	S						
Aza 30d (5)	Aza					BrdU	S		

Figure 9 Hippocampal proliferation after antimitotic treatment. (a) Number of BrdU-positive cells in animals untreated with KA, at different times after azacytidine treatment as compared to animals injected with saline. (b) Number of BrdU-positive cells in animals treated with KA only or with KA after azacytidine treatment as compared to animals treated with saline. In the DG, after 4 days (d) of azacytidine, KA-induced cell proliferation was dramatically reduced. When KA was administered 30 days after the antimitotic, cell proliferation was similar to that induced by KA in mice not treated with azacytidine. In CA1 azacytidine treatment weakly affected the proliferation induced by KA; p values, significantly different as compared to * dentate gyrus ($p < 0.05$), † control ($p < 0.01$), or ζ KA-treated ($p < 0.01$) mice. Data represent the media of three to nine different animals per group. (c) Schematic representation of the timing of the different treatments (Aza, azacytidine; d, days; S, day of sacrifice).

CA1 and DG were reduced to around 20% (data not shown), but completely recovered after 30 days. No significant differences between the two fields were observed. In animals treated with the antimitotic 4 days before, KA still enhanced cell proliferation [Fig. 9(b)]. However, as compared to animals treated with KA only (no azacytidine), the number of proliferating cells was dramatically reduced, particularly in the DG. The CA1 field displayed a better capacity to increase proliferation. Thirty days after azacytidine, KA displayed similar capacities to induce cell proliferation as compared to nonazacytidine animals.

DISCUSSION

The present data strongly suggest the presence of two different types of precursors cells in adult hippocampus. They have distinct distribution (CA1 vs. DG), differently stain for precursor cell markers, and display differential functional properties in terms of mitogens (EGF vs. FGF-2) that activate their proliferation, multipotency, and expansion-renewal.

Precursors cells isolated from CA1 dissected fields proliferated in the presence of EGF or FGF-2 to form spheres, were highly expandable, and were multipotent and generate neurons, astrocytes, and oligodendrocytes. They may be considered therefore as neural stem cells. The analysis of *in vivo* preparations and organotypic explants revealed the presence of BrdU, Nestin, and Mash-1-immunopositive cells in CA1. These precursors displayed a high capacity of expansion as BrdU incorporation and Nestin and Mash-1 immunoreactivity rapidly increased after seizures, even after a treatment with antimitotic drugs such is azacytidine, suggesting that CA1 precursors rapidly

regenerate *in vivo* its own population. These cells apparently distribute not only within the periventricular zone but the stratum oriens as well, thus suggesting that expandable precursors also reside within the hippocampal parenchyma. Finally, our data also demonstrate the genesis of new neurons and glial cells, mainly after seizures, cells that distribute within the deeper layers of CA1, which is compatible with the presence of multipotent precursors in CA1. However, Seaberg and Van der Kooy (2002) suggested that neurospheres grown from adult hippocampus derive from posterior periventricular progenitors rather than from precursors present within the parenchyma. Their conclusion resided in the results obtained after microdissection of hippocampal layers, a procedure that may damage the region of interest. If only the more ventricular precursors share the properties of stem cells, the remaining cells should be more committed precursors that may originate neurons and/or glial cells (which in turn may also be Nestin immunoreactive; Filippov et al., 2003), or would-be cells originated from periventricular precursors that rapidly migrate to colonize the hippocampal parenchyma.

In the DG, precursor cells also express Nestin but only a small proportion of DG BrdU-positive cells express Mash-1, suggesting the presence of two subsets of proliferating precursors. *In vitro* analysis revealed that DG precursors scarcely respond to EGF and produce around eight times less spheres than CA1 cells. This agrees with the observation that EGF treatment of organotypic explants raises BrdU incorporation by only 10 to 20% (not statistically significant). Though these EGF responsive cells may be reminiscent of CA1 precursors, subclonal analysis revealed that they are less expandable. This agrees with the observation that *in vivo* they expand less than CA1 precursors and recover worst after antimitotic treatment. Furthermore, isolated DG precursors are less multipotent than CA1 precursors and mainly produce glial cells. Though the dentate gyrus lacks of FGF-2 responsive neurosphere-forming cells, treatment of organotypic explants with FGF-2 clearly increases BrdU incorporation and Nestin expression. However, this effect is not restricted to a hippocampal field or layer and may just represent the mitogenic effect of this factor on glial cells (Perraud et al., 1990). In conclusion, these data reveal that DG precursors are less "stem cell" in nature than CA1 and display different properties.

The first difference between DG and CA1 precursors *in vitro* is the responsiveness to FGF-2. Two hypotheses would explain it: DG precursors do not express FGF receptors or FGF-2 mitogenic effects on DG precursors require additional factors. There is a relatively large amount of literature demonstrating that granule cell proliferation may be modulated *in vivo* by serotonin (Brezun and Daszuta, 2000a,b), sexual steroids (Tanapat et al., 1999), adrenal steroids (Cameron and Gould, 1994), thyroxin (Hadj-Sahraoui et al., 2000), insulin (Aberg et al., 2000), and glutamate (Gould et al., 1997). Although it is presently unknown if these factors act directly on granule cell precursors, it is plausible that they or other factors contribute to modulate FGF receptor expression or function. In fact, recent data suggest that FGF-2 mitogenic effects on granule cells would require a glycosilated form of cystatin (Taupin et al., 2000). Therefore, we may conclude that FGF-2 is a mitogen for both DG and CA1 precursors but that only granule cell precursors would require additional factors.

DG and CA1 precursors displayed distinct expansion-renewal properties *in vivo* and *in vitro*. Granule cell precursors have a lower expansion-renewal potential than CA1. This is not surprising when considering that neurogenesis is more important in the former area (i.e., in control mice the mean number of BrdU+NeuN+ cells in the subgranular zone represented up to 70% of total BrdU-positive cells while in the stratum oriens they concerned only 15% of total BrdU-positive cells). It may be suggested that DG precursors are more committed *in vivo* in the production of new neurons than CA1 precursors, which are more quiescent and more engaged in the maintenance of their own population. Antimitotic treatment that depletes both populations is consequently associated with a better recovery of the precursor population in CA1 but with a more restrained recovery in DG. In agreement with this suggestion it has been recently shown that X-ray irradiation was associated in rat hippocampus with a long-lasting (up to 120 days) reduction of granule cell proliferation (Tada et al., 2000), suggesting that granule cell precursors were unable to repopulate the subgranular zone. However, a more recent report demonstrated that the treatment of Sprague-Dawley adult rats with the DNA methylating agent methylazoxymethanol acetate resulted in a clear-cut reduction of granule cell proliferation that recovered after 30 days (Shors et al., 2001) as in the present study.

Our *in vitro* analysis revealed that in the absence of other signals, DG precursors are less multipotent than CA1 precursors and that most of the DG isolated clones did not produce neurons. This sounds contradictory with *in vivo* data depicting that DG precursors produce high numbers of neurons. We may conclude that in the DG, EGF recruits glial precursor cells. The precursor responsible for the ongoing DG neurogenesis would not express EGF receptors. More recently,

Seaberg and Van der Kooy (2002) reported that isolated primary cells derived from DG generate quite small numbers of spheres in the presence of EGF+FGF-2, none of them being multipotent. These authors, however, succeeded in isolating small clumps of cells grown under EGF+FGF-2 that produced neurons only. It is thus plausible that granule cell precursors are FGF-2 responsive (Taupin et al., 2000), but that they simply do not have the properties of stem cells (as cerebellar granule cell precursors; Alder et al., 1996; Jankovski et al., 1996). The elegant studies by Altman and Bayer (1990a,b) about the formation of the DG provide some hints for the understanding of the present issue. Using tritiated thymidine these authors have shown that hippocampal precursors located within a ventricular indentation by E18 migrated to the intermediate zone where they originated a second proliferative field, the DG itself. Hence, granule cells are originated from a postmigratory proliferating second generation of precursors.

CA1 and DG precursors are responsible for the genesis of new neurons and glial cells in these fields. In the dentate gyrus, new neurons apparently succeed a functional integration (Van Praag et al., 2002), and may play a major role in associative learning tasks (Gould et al., 1999). The role for CA1 neurogenesis remains elusive and more should be done to clarify which cell type it concerns and to investigate if these new neurons succeed a functional integration. Recently it has been shown that CA1 precursors may produce new pyramidal cells (Nakatomi et al., 2002). Interestingly, these authors demonstrated that in ischemic animals, the intraventricular coinfusion of EGF+FGF-2 resulted in a dramatic increased proliferation of precursors that were able to restore, at least partially, the damaged CA1 pyramidal cell layer. In these animals, regenerated neurons integrate the existing neuronal circuitry and contribute to ameliorate the ischemia-induced neurological deficits. In the present article, newly formed neurons (NeuN+ or calbindin+) distributed mainly throughout the stratum oriens. The analysis of animals sacrificed 15 days after BrdU did not reveal any major sign for cell migration, suggesting that proliferating cells remain more or less at the place in which they were generated. It is thus plausible that these newly formed neurons are GABA interneurons, which localize essentially on neuropile layers (also see Rietze et al., 2000). However, these cells did not express any specific marker for interneurons 7 to 15 days after the last BrdU injection, including GAD, somatostatin, parvalbumin, or cholecystokinin (results not shown). It may be that more time is required for a full differentiation of newly formed neurons. It is worth it to stress that seizures might induce cell death of interneurons, mainly in the oriens, without essentially affecting CA1 pyramidal cells (Cossart et al., 2001). Therefore, it might be that the increased genesis of neurons observed in CA1 constitutes an effort to replace degenerating neurons and to restore the inhibitory feedback. The present data reinforce the suggestion by Nakatomi et al. (2002) that CA1 precursors may constitute an intrinsic therapeutic tool for repairing a damaged CA1 field after ischemia, epilepsy, or other degenerative diseases known to affect this hippocampal field. Our results, depicting a quite selective effect of EGF rather than FGF-2 on the recruitment of CA1 multipotent precursors, strongly support this factor as a good candidate to enhance the genesis of CA1 neurons from endogenous precursors.

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