New neurons in the dentate gyrus are involved in the expression of enhanced long-term memory following environmental enrichment

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Abstract

Although thousands of new neurons are continuously produced in the dentate gyrus of rodents each day, the function of these newborn cells remains unclear. An increasing number of reports have provided correlational evidence that adult hippocampal neurogenesis is involved in learning and memory. Exposure of animals to an enriched environment leads to improvement of performance in several learning tasks and enhances neurogenesis specifically in the hippocampus. These data raise the question of whether new neurons participate in memory improvement induced by enrichment. To address this issue, we have examined whether the increase in the number of surviving adult-generated cells following environmental enrichment contributes to improved memory function. To this end, neurogenesis was substantially reduced throughout the environmental enrichment period using the antimitotic agent methylazoxy-methanol acetate (MAM). Recognition memory performance of MAM-treated enriched rats was evaluated in a novel object recognition task and compared with that of naïve and nontreated enriched rats. Injections of 5-bromo-2′-deoxyuridine were used to label dividing cells, together with double immunofluorescent labelling using glial or neuronal cell-specific markers. We found that enrichment led to improved long-term recognition memory and increased hippocampal neurogenesis, and that MAM treatment during environmental enrichment completely prevented both the increase in neurogenesis and enrichment-induced long-term memory improvement. These results establish that newborn cells in the dentate gyrus contribute to the expression of the promnesic effects of behavioural enrichment, and they provide further support for the idea that adult-generated neurons participate in modulating memory function.

Introduction

Substantial numbers of new neurons are continuously produced in the adult vertebrate brain, ranging from birds to primates (Gage, 2002; Gould & Gross, 2002). These new neurons are produced from progenitor cells located in restricted brain regions, including the subgranular zone of the dentate gyrus of the hippocampal formation. Daughter cells, generated locally at the border between hilus and granule cell layer, migrate into the granule cell layer where they develop morphological and biochemical characteristics of mature neurons (Stanfield & Trice, 1988; Hastings & Gould, 1999; Markakis & Gage, 1999). They receive synapses, extend axonal connections to CA3 and become functionally integrated into existing neuronal circuitries (van Praag *et al*., 2002). Although thousands of new neurons are produced in the dentate gyrus of rodents each day (Cameron & McKay, 2001), the function of these new cells is still unclear. Several reports have provided correlational evidence that adult hippocampal neurogenesis is involved in learning and memory (Gould *et al*., 1999b). For example, training on hippocampal-dependent learning tasks specifically enhances survival of adult-generated neurons in the rat dentate gyrus (Gould *et al*., 1999a) and, conversely, the reduction of neurogenesis by a cytostatic drug impairs associative hippocampal-dependent, but not hippocampal-independent, learning (Shors *et al*., 2001).

Several factors have been shown to regulate neurogenesis in mammals (Gould *et al*., 2000; Zhao *et al*., 2004). For decades, exposure to an enriched environment, consisting of housing groups of animals together in a complex environment with various toys providing more opportunity for learning and social interaction than standard laboratory living conditions (Rosenzweig, 1966), has been shown to enhance behavioural performance in various learning tasks and increase the number of dendritic spines and synapses in cortex and hippocampus (Rampon & Tsien, 2000; van Praag *et al*., 2000). More recently, studies have reported that enrichment enhances survival of newly generated neurons in adult rodents (Kempermann *et al*., 1997, 1998a,b; Nilsson *et al*., 1999; van Praag *et al*., 1999b). In naïve animals, the majority of new hippocampal neurons degenerate within a few weeks after cell division (Gould *et al*., 1999a; Biebl *et al*., 2000), suggesting that exposure to environmental complexity acts as a survival-promoting factor. What is the contribution of neurogenesis to the beneficial effect of enrichment on cognitive functions?

Here, we addressed this issue by measuring the extent to which increased hippocampal neurogenesis following enrichment contributes to the enhancement of hippocampal function. To this end, we used an antimitotic agent to reduce enrichment-induced neurogenesis throughout the enrichment period and assessed memory performance of rats with intact or reduced neurogenesis in a novel object recognition task. This behavioural paradigm, adapted from human studies designed to probe declarative memory (Manns *et al*., 2003), is based on the spontaneous preference of rodents for novelty and their ability to remember previously encountered objects (Ennaceur & Delacour,

1988). We used 5-bromo-2′-deoxyuridine (BrdU) to label and quantify dividing hippocampal cells and examined whether enrichment alters the fate of these cells using confocal microscopy analysis of glial and neuronal cell-specific markers.

Materials and methods

Animals and housing conditions

Adult male Sprague Dawley rats (Charles River, Orléans, France), weighing 220–250 g were housed in pairs in a temperature-controlled animal facility with a 12-h light–dark cycle and had access to food and water *ad libitum*. All experiments were performed in strict accordance with the recommendations of the European Union (86/609/EEC) and the French National Committee (87/848). Prior to experiments, the rats were left undisturbed for 7 days and were randomly assigned to one of four experimental conditions: saline–naïve ($n = 16$), methylazoxy-methanol acetate (MAM)–naïve ($n = 10$), saline–enriched ($n = 15$) and MAM–enriched ($n = 11$). Animals in the two naïve groups were held by pairs in standard laboratory cages whereas rats of the two enriched groups were placed in groups of four to six in a complex enriched environment for 3 h/day for 14 days. This enriched environment consisted of a large box ($1.5 \times 0.8 \times 0.8$ m) containing various toys, wooden blocks, climbing platforms, plastic tubes and small houses as described previously (Rampon *et al.*, 2000). One exception is that there was no running wheel. Toys were rearranged and renewed every day to favour animals' exploratory behaviour. In the box, animals had free access to food and water.

Drugs and injection protocol

From day 1 to day 14 (Fig. 1), animals from MAM–enriched and MAM–naïve groups were given an injection of methylazoxymethanol acetate (MAM; 5 mg/kg, in 0.9% NaCl, subcutaneous), whereas rats from saline–enriched and saline–naïve groups were given an injection of the vehicle solution (0.9% NaCl, subcutaneous). MAM is an antimitotic DNA methylating agent (Johnston & Coyle, 1979) which
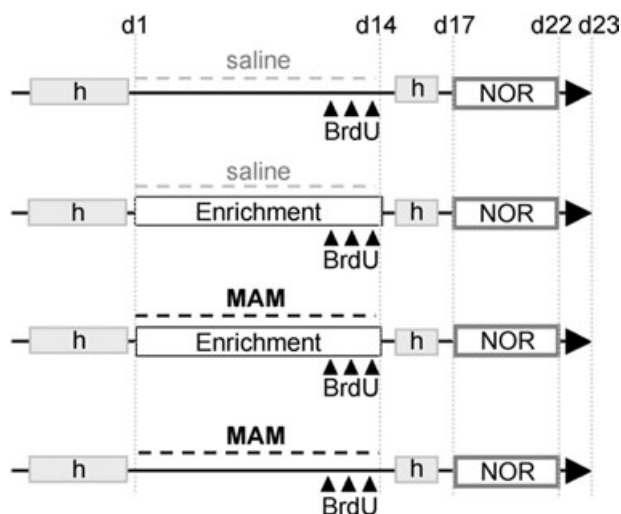

FIG. 1. Schematic representation of the experimental design and time-structure of the protocol (see Materials and methods for details). Saline or MAM was injected daily during the enrichment period, days 1–14 (d1 to d14) or an equivalent period in nonenriched rats. BrdU was injected on days 10, 12 and 14 (arrowheads) and animals were tested in the novel object recognition (NOR) task on days 17–22 after habituation sessions (h).

when injected at 5 mg/kg reduces hippocampal neurogenesis without any toxic effect in rats (Shors *et al.*, 2001, 2002). Injections were given prior to exposure to the enriched environment. To evaluate hippocampal neurogenesis, all rats were injected with BrdU (100 mg/kg, intraperitoneal; Sigma) on days 10, 12 and 14 of the enrichment period (Fig. 1). The thymidine analogue BrdU is incorporated into the genetic material upon mitotic division, after which it can be detected in the daughter cells by immunohistochemistry (del Rio & Soriano, 1989; Nowakowski *et al.*, 1989).

Object recognition task

Behavioural testing began on day 17 and lasted for 6 days (Fig. 1). The rats were handled and habituated to the empty open field ($100 \times 100 \times 40$ cm) for 5 days preceding the enrichment period and were given a further 2 days of habituation on days 15 and 16 prior to behavioural testing. For the acquisition phase, rats were placed into the open field containing two different objects, which they had never seen, and were allowed to explore for a single 4-min session during which the time spent exploring each object was recorded. Recognition memory was tested 1, 24 and 48 h later in a 4-min test session during which one object used during training was replaced by a novel object. Each rat was tested at the three retention intervals (in a counterbalanced order) with different triplets of objects each time. The objects, which varied in shape and colour and were made of glass, cardboard and plastic, were fixed to the floor, 15 cm from the walls, and cleaned thoroughly between trials to ensure the absence of olfactory cues. The objects had been tested before in naïve rats to ensure an equivalent level of spontaneous preference. In addition, the nature and spatial position of the object that was changed during the test session were counterbalanced within groups and within delays, in order to avoid any bias due to a preference that rats may have for a given object or its position in the open field. Object exploration was scored when the rat was within a 1-cm zone circumscribing the object, with its nose facing it (Clark *et al.*, 2000). All rats reached the criteria of a minimum of 20 s total object exploration time for each presentation. Measurement of the time spent exploring the novel object (preference index) was expressed as a percentage of the exploration time of the novel object related to the total exploration time for both objects during the test phase. Statistical analysis of object exploration time and preference index was performed with repeated-measures, one-way and two-way ANOVA followed by the Newman–Keuls *post hoc* analysis. Student's *t*-test was used to determine whether preference index was significantly different from chance (50%).

Tissue preparation

The day following behavioural testing (day 23, 9 days after the last BrdU injection), the animals were anaesthetized with an overdose of sodium pentobarbital and perfused transcardially with 0.1 M phosphate buffer (PB; pH 7.4) followed by 4% paraformaldehyde in 0.1 M PB, at 4 °C. The brains were left in the fixative overnight, and then transferred into 30% sucrose. Coronal sections (30 μm) were serially cut using a cryostat and stored at −20 °C in cryoprotectant until processed for immunohistochemistry.

Immunohistochemistry

For peroxidase single-immunolabelling of BrdU, free-floating sections were treated in order to denature the DNA (2 N HCl for 40 min at room temperature) and rinsed in 0.1 M boric acid (pH 8.5). After several rinses in PB containing 0.9% NaCl and 0.25% Triton X-100

(PBST), sections were incubated overnight in primary mouse mono-clonal anti-BrdU (1 : 300; Roche Diagnostics, Indianapolis, IN, USA) in PBST containing 5% normal goat serum. The next day, sections were incubated in biotinylated goat antimouse antisera (1 : 200; Sigma) for 90 min, rinsed and incubated in avidin–biotin–peroxidase complex (1 : 300; Vectastain Elite Kit, Vector, Burlingame, CA, USA) for 90 min. Finally, they were reacted for peroxidase detection in a solution of 3,3′-diaminobenzidine (0.5 mg/mL; Sigma) containing 0.003% H_2O_2 and 0.06% nickel ammonium sulphate. Sections were mounted onto slides, counterstained with Nuclear Fast Red (Vector) and cover-slipped. Sections incubated without the primary antibody remained virtually free of immunostaining and served as controls. For fluorescence immunolabelling of BrdU combined with markers of glial cells (glial fibrillary acidic protein, GFAP) or of both immature and mature neurons (class III β-tubulin, Tuj-1), sections were denatured as described above, followed by overnight incubation in rat monoclo-nal anti-BrdU (1 : 400; Harlan Sera-lab, Loughborough, England) alone or simultaneously with a polyclonal anti-GFAP (1 : 5000; Dako, Glostrup, Denmark). On the next day, they were incubated in biotinylated goat antirat antisera (1 : 400; Vector) for 90 min. They were then rinsed and incubated in Streptavidin Alexa-568 (1 : 1000; Molecular Probes, Eugene, OR, USA) alone or with Alexa-488 goat antirabbit antisera (1 : 250; Molecular Probes) for 90 min. Sections stained with BrdU alone were incubated overnight in mouse mono-clonal anti-Tuj-1 (1 : 1300; BabCo, Richmond, CA, USA), rinsed and incubated in goat antimouse highly cross-absorbed antisera conjugated to Alexa-488 (1 : 250; Molecular Probes) for 90 min. After several rinses, sections were mounted, dried and counterstained with DNA dye bisbenzimid (Hoechst 33342, Sigma; 1 μg/mL), and cover-slipped under fluoromount-G (SouthernBiotech, Birmingham, AL, USA). Antibodies were tested with the appropriate negative controls (recip-rocal omission of primary and secondary antibodies).

Quantification and image analysis

Quantification was conducted in a blind manner. The number of BrdU-labelled cells in the left and right dentate gyrus was estimated by using a design-based stereological sampling on every twelfth section (360 μm apart) through the rostrocaudal extent of the hippocampus. As BrdU-positive cells are relatively rare in the dentate gyrus, no counting frames were used but rather every BrdU-positive cell within the granule cell layer and adjacent subgranular zone, defined as a two-cell-body-wide zone along the border of the granule cell layer, was counted through a ×100 objective using a Nikon microscope. Nuclei intersecting the uppermost focal plane were excluded from the count in order to avoid oversampling error. On the same sections counterstained with Nuclear Fast Red, the corresponding sampled surfaces of the granule cell layer and subgranular zone were measured by tracing the area with the Mercator morphometric system (Explora Nova) through a ×10 objective. The reference volume was determined as the sum of the traced areas multiplied by the distance between sampled sections (360 μm). The density of BrdU-positive cells was then calculated by dividing the number of BrdU-positive cells by granule cell layer sectional volume. The total number of BrdU-positive cells was estimated by multiplying these densities by the reference volume. To determine the relative distribution of the phenotypes of newborn cells, a series of every twelfth section was double-labelled for BrdU and Tuj-1 (Geisert & Frankfurter, 1989; Menezes & Luskin, 1994) (five animals in each group, randomly selected) or for BrdU and GFAP (four animals in each group, randomly selected), as described above. For each animal, a minimum of 50 BrdU-labelled cells were randomly selected throughout the dentate gyrus and analysed for coexpression of

BrdU and the neuronal-type specific marker Tuj-1 or for coexpression of BrdU and GFAP, using a confocal laser-scanning microscope equipped with krypton–argon 488–568 lasers (Bio-Rad Laboratory MRC1024ES, 510 LSM, Zeiss, Germany). Each BrdU-positive cell was analysed in its entire z-axis, with 0.5-μm step intervals using a ×60 oil-immersion objective. Labelled cells were rotated in orthogonal planes (x and y) to verify unequivocally double-labelling and to exclude false double-labelling due to overlay of signals from different cells (Kuhn et al., 1997; Gould et al., 2001). All analyses were carried out in sequential scanning mode in order to rule out cross-bleeding between detection channels. The number of double-labelled cells in each category was determined. Ratios of BrdU-positive cells colabelled with Tuj-1, GFAP or neither of these phenotypic markers and the mean ± SEM were calculated for each group. Optical stacks of images were obtained for figures. Images were processed using Confocal Assistant software 4.02 (Todd Clark Brelje) and Adobe Photoshop software 7.0 (Adobe System). Statistical significance was determined with ANOVA followed by Newman–Keuls post hoc analysis.

Results

Neurogenesis

Newborn cells in the adult rat dentate gyrus can be quantified by immunostaining of BrdU incorporated into nuclei of dividing cells (Gage, 2002; Gould & Gross, 2002). In our study, BrdU labelling was studied 9–13 days after BrdU injections (Fig. 1), corresponding to the survival period after proliferation of the progenitors, when the newborn cells develop differentiated phenotypes (Kempermann et al., 2003). The morphological appearance of BrdU-labelled nuclei was dark and round-shaped, frequently with the typical morphology of granule cell nuclei (Fig. 2a–d). The comparison of the reference volume revealed that neither housing conditions nor drug treatment had any significant effect (Newman–Keuls, $P > 0.05$ in each case, data not shown). The total number of BrdU-positive cells in the four groups are shown on Fig. 2e. A two-way ANOVA revealed a significant main effect of enrichment ($F_{1,24} = 5.47$, $P < 0.05$) and a significant main effect of MAM treatment ($F_{1,24} = 24.18$, $P < 0.0001$). The effect of environmental enrichment on dentate gyrus neurogenesis was evaluated by comparing the total number of BrdU-positive cells in the dentate gyrus of saline–naïve ($n = 8$) and saline–enriched rats ($n = 8$). As expected, we found that enrichment led to a large and significant increase in the number of BrdU-labelled cells in the dentate gyrus when compared to saline-injected, naïve rats (saline–enriched, 4582.5 ± 523.7 BrdU-positive cells; saline–naïve, 2715 ± 318.5 BrdU-positive cells; Newman–Keuls, $P < 0.005$; Fig. 2a, c and e). These results indicate that 3 h of daily housing in the enriched environment for 14 days is a condition sufficient to promote newborn cell survival. We then used the DNA methylating agent MAM to reduce proliferation (Johnston & Coyle, 1979; Shors et al., 2001). MAM was administered at the same dose as that used by Shors et al. (2001), who showed that 14 days of MAM treatment does not alter gross neurophysiological responsiveness in the hippocampus, levels of motor activity, or corticosterone blood levels. Quantification of BrdU-labelled cells revealed that MAM treatment in naïve rats ($n = 6$) reduced the number of newborn cells in the dentate gyrus by $> 50\%$ compared to saline–naïve rats (MAM–naïve, 1388 ± 344.1 BrdU-positive cells; Newman–Keuls $P < 0.05$; Fig. 2e). This confirms that MAM treatment produced a substantial reduction in newborn cells in the dentate gyrus. In the enriched groups, statistical analysis revealed that the total number of BrdU-labelled cells in MAM-treated enriched rats ($n = 6$, 1580 ± 493.5 BrdU-positive cells; Fig. 2b,d and e) was
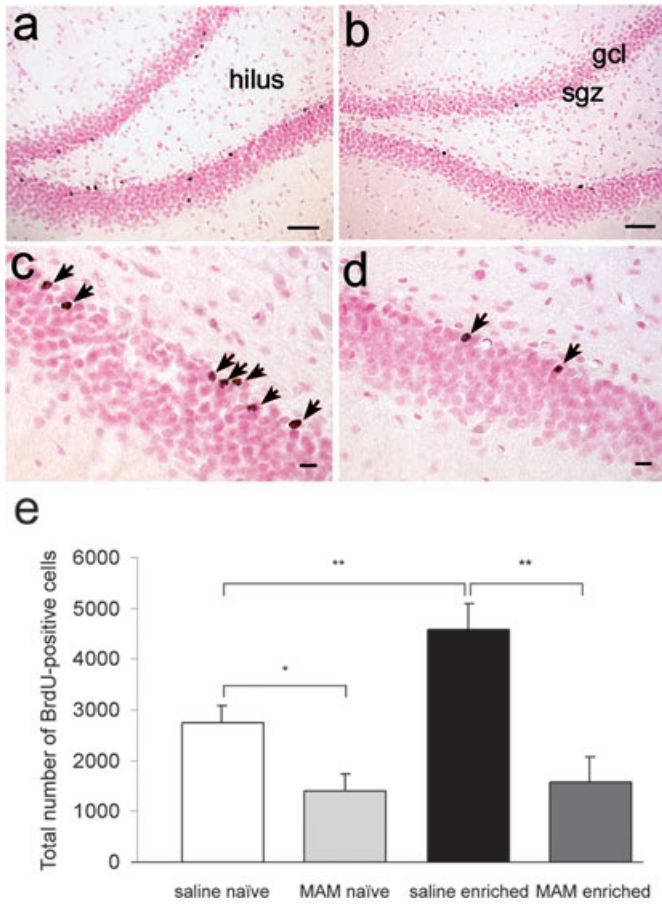
FIG. 2. Effect of environmental enrichment and of the antimitotic MAM treatment on dentate gyrus neurogenesis in adult rats. (a–d) Light photomicrographs of Nuclear Fast Red-stained sections showing the distribution of BrdU-immunoreactive nuclei in the dentate gyrus of representative rats from (a) the saline–enriched and (b) the MAM–enriched groups; sgz, subgranular zone; gcl, granule cell layer. (c-d) Higher magnification photomicrographs illustrate (d) the reduced number of newborn cells (arrows) in the gcl of enriched rats treated with MAM, compared to (c) saline–enriched rats. (e) Quantitative data are expressed as total number of dentate gyrus BrdU-labelled cells (see Materials and methods). Bars represent mean ± SEM. Enrichment resulted in a marked increase in the total number of BrdU-labelled cells compared to the saline–naïve group. MAM treatment significantly reduced the total number of BrdU-positive cells in naïve rats and abolished the increase in BrdU-positive cells induced by enrichment. *$P < 0.05$, **$P < 0.005$. Scale bars, 50 μm (a and b), 10 μm (c and d).

drastically reduced compared to that of the saline–enriched rats (Newman–Keuls, $P < 0.005$; Fig. 2a, c and e). MAM treatment administered during enrichment induced >65% reduction of the number of BrdU-labelled cells (Fig. 2e). Residual numbers of BrdU-labelled cells were approximately the same in both MAM-treated groups regardless of their housing conditions (Newman–Keuls, $P > 0.05$). Thus, these results confirm that behavioural enrichment promoted survival of adult-generated cells in the dentate gyrus and show that MAM treatment completely abolished enrichment-induced dentate gyrus neurogenesis.

Effect of enrichment and MAM on differentiation of newborn cells

Differentiation of BrdU-positive cells was examined by determining the phenotype into which these cells had differentiated, based on
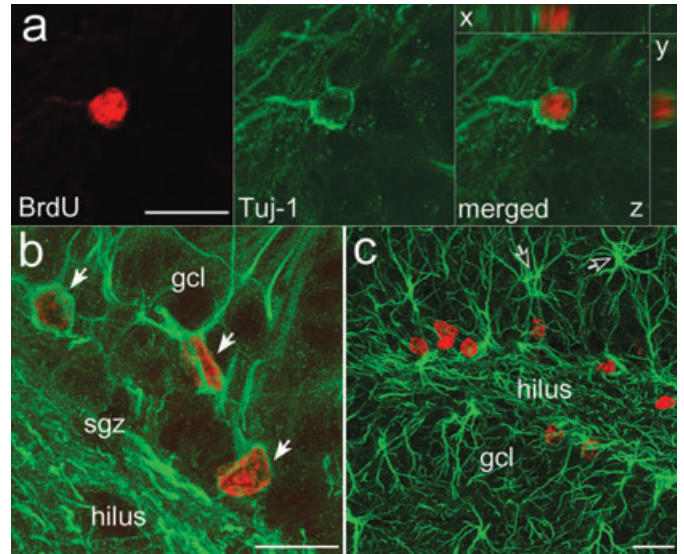

FIG. 3. Phenotypic analysis of BrdU-labelled cells. (a) Representative confocal laser scanning microscope image depicting a cell double-labelled for BrdU (red nuclear stain, left) and for the neuronal marker Tuj-1 (green cytoplasmic stain, middle) in the dentate gyrus. On the right (merged), cytoplasmic Tuj-1 (green) expression engulfs nuclear BrdU labelling (red). Co-localization of BrdU and Tuj-1 was systematically verified by rotating the image in orthogonal planes (x, y) and by z-sectioning at 0.5-μm intervals. (b) The majority of newborn neurons labelled with BrdU (red) in the dentate gyrus coexpressed Tuj-1 (green), a marker for both immature and mature neurons (arrows). (c) BrdU-positive cells (red) were rarely double-labelled with the astroglial marker GFAP (green), independently of experimental conditions. As an example, two GFAP-single-labelled cells are indicated by arrows. sgz, subgranular zone; gcl, granule cell layer. Quantitative analysis is presented in Table 1. Scale bars, 20 μm (a–c).

immunofluorescent double-labelling for BrdU and the neuronal-type marker Tuj-1, and for BrdU and the glial marker GFAP. We used confocal microscopy to count the number of double- or single-labelled BrdU-positive cells in the dentate gyrus. Double-labelled cells for BrdU and Tuj-1 appeared with a red nucleus surrounded by green labelling of the cytoskeleton visible in the three orthogonal planes (Fig. 3a). Their immature status revealed by immunoreactivity to both BrdU and Tuj-1 is supported by their distribution pattern in the inner third of the granule cell layer, indicating that they did not achieve their migration (Fig. 3b). In all four experimental groups, we found that the large majority of BrdU-positive cells also expressed Tuj-1 immunoreactivity (Table 1), while a minority of BrdU-positive cells were immunoreactive for the glial marker GFAP (Fig. 3c and Table 1). The remaining BrdU-positive cells in the dentate gyrus were not immunoreactive to either of the cell-specific markers and were probably

TABLE 1. Quantitative confocal analysis of newborn cell differentiation in the different experimental conditions

Group	BrdU–Tuj-1	BrdU–GFAP	BrdU only
Saline–naïve	79.9 ± 3.93%	2.5 ± 0.96%	18.6 ± 4.39%
Saline–enriched	84.9 ± 3.25%	4.0 ± 0.04%	11.9 ± 4.10%
MAM–naïve	73.8 ± 3.39%	3.5 ± 0.50%	23.1 ± 4.03%
MAM–enriched	58.2 ± 3.67%*	5.2 ± 0.82%	38.4 ± 4.63%*

Percentage of BrdU-labelled cells immunoreactive for the neuronal marker Tuj-1, the glial marker GFAP or none of these markers (50 BrdU-positive cells were counted per animal for each marker). Data are group mean ± SEM. *$P < 0.05$ compared to saline–enriched rats.

undifferentiated progenitors or glial cells such as microglia or oligodendrocyte pools that were not labelled in the present study.

In saline groups, enrichment did not affect the differentiation of newly generated cells towards either neuronal or glial fate (79.9 ± 3.93% and 84.9 ± 3.25% of neuronal differentiation and 2.5 ± 0.96% and 4 ± 0.04% of glial differentiation in saline–naïve and saline–enriched rats, respectively; Newman–Keuls, $P > 0.05$ in each case; Fig. 3d and Table 1). Similarly, MAM treatment in naïve rats had no effect on neuronal or glial differentiation (73.8 ± 3.39% of double-labelled BrdU–Tuj-1 cells and 3.5 ± 0.5% of double-labelled BrdU–GFAP cells in MAM–naïve rats, $P > 0.05$ in each case). Surprisingly, we found a smaller proportion of BrdU–Tuj-1 cells in MAM–enriched rats than in saline–enriched rats (58.2 ± 3.67% and 84.9 ± 3.25%, respectively; Newman–Keuls $P < 0.05$; Table 1) whereas these groups exhibited a similar proportion of newborn cells expressing GFAP (5.2 ± 0.82% and 4 ± 0.04%, respectively; $P > 0.05$), suggesting that MAM treatment administered during enrichment might slow down cell differentiation or increase death of newborn neurons. These results indicate that neither the environmental conditions of enrichment nor MAM treatment affected differentiation of newly generated cells towards neuronal or glial fate, but the combination of both factors, i.e. MAM treatment in a condition of increased neurogenesis, tended to reduce neuronal differentiation.

Environmental enrichment improves long-term recognition memory

To test the effect of enrichment on memory, rats were tested in a novel object recognition task during the last 5 days of the experimental protocol (Fig. 1) and recognition memory was assessed at retention intervals of 1, 24 and 48 h in independent replicates in each rat. During the acquisition phase, the average total time spent in object exploration over the three replicates was slightly reduced in saline–enriched group compared to saline–naïve group (65.7 ± 6 s and 82.7 ± 6.2 s, respectively; data not shown) but this difference was not significant (Newman–Keuls, $P > 0.05$). When tested at the short retention interval of 1 h following the acquisition phase, both saline–naïve and saline–enriched rats spent significantly more time exploring the novel object than the familiar object (62.3 ± 3%; $t_{15} = 4.16$, $P < 0.005$ and 65.4 ± 3.9%; $t_{12} = 3.94$, $P < 0.005$, respectively; Fig. 4). The two groups exhibited the same level of performance at this short delay. At the longer retention intervals, saline–naïve rats spent an equal amount of time exploring each object, with a level of exploration of the novel object not significantly different from chance (54.3 ± 2.4% and 48.4 ± 3.3% at 24 and 48 h, respectively; $P > 0.05$ in each case; Fig. 4). In contrast, as illustrated in Fig. 4, saline–enriched rats maintained a significant preference for the novel object at 24 and 48 h (64.5 ± 1.7%; $t_{13} = 8.44$, $P < 0.0001$ and 62.1 ± 1.3%; $t_{14} = 10.48$, $P < 0.0001$, respectively), indicating that they remembered the objects for at least 48 h. A repeated-measures ANOVA on retention performance in enriched and nonenriched saline groups confirmed an overall beneficial effect of enrichment on memory ($F_{1,27} = 16.13$, $P < 0.005$) and the Newman–Keuls *post hoc* analysis showed a significant difference between the two groups at both 24 and 48 h ($P < 0.005$ at both delays; Fig. 4). These results show that whereas naïve rats had a rapidly decaying memory of the objects in this protocol based on a single, brief (4 min) exposure to the objects, enriched rats were able to form a stable long-term memory of the
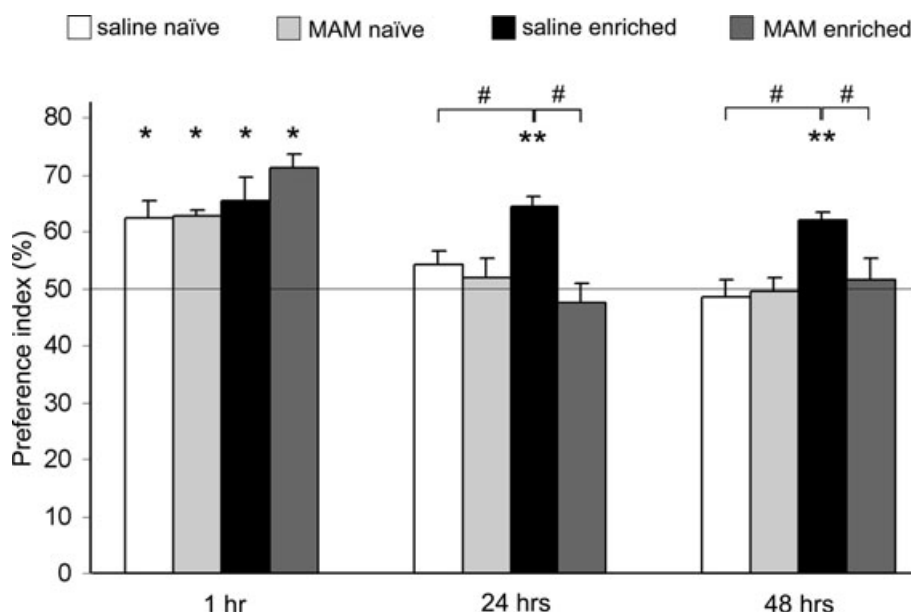

FIG. 4. Effect of environmental enrichment and of the depletion of enrichment-induced neurogenesis on recognition memory. Rats in the different conditions were briefly exposed to two objects and recognition memory was tested in independent replicates 1, 24 and 48 h later with a familiar object replaced by a novel object. Retention performance is expressed as the group mean (± SEM) preference index (percentage time spent exploring the novel object over the total time of object exploration). Rats from all groups spent significantly more time exploring the novel object at the 1 h delay, showing good short-term memory (left panel). Saline-injected, nonenriched rats (open bars, $n = 16$) showed no preference for the novel object at 24 or 48 h in this protocol with a very brief 4-min exposure to objects during the sample phase (central and right panels). In contrast, saline–enriched rats (solid bars, $n = 15$) explored preferentially the novel object at the two long-term delays. Thus, enrichment resulted in a marked facilitation of long-term recognition memory. MAM treatment in naïve rats (light bars, $n = 10$) did not affect recognition memory at any time point, compared to saline–naïve rats (open bars). MAM-treated enriched rats (dark bars, $n = 11$) spent significantly more time exploring the novel object at the 1 h delay (left panel), indicating normal short-term memory, but long-term memory for objects was impaired compared to saline–enriched rats (solid bars) when tested at 24 or at 48 h (middle and right panel), indicating that MAM treatment in enriched rats completely abolished the beneficial effect of enrichment on long-term recognition memory. The horizontal line represents equal exploration of the novel and familiar objects. *$P < 0.005$, **$P < 0.0001$, #$P = 0.005$.

objects. Although enriched rats spent slightly less time exploring the objects during the acquisition phase, the enrichment protocol resulted in an important improvement of long-term recognition memory.

MAM treatment prevents enrichment-induced long-term memory improvement

We then tested the effect of MAM treatment on recognition memory in enriched and nonenriched rats. First, when compared to the saline–naïve group, we found that naïve rats treated with MAM spent an equal amount of time exploring the objects during the acquisition phase (82.7 ± 6.2 and 82.4 ± 4.5 s, respectively; Newman–Keuls, $P > 0.05$; data not shown), indicating that MAM injections had no discernible effects on motor activity or on their natural tendency for object exploration. At the 1-h retention delay, both groups of rats showed a significant preference for the novel object ($62.3 \pm 3\%$; $t_{15} = 4.16$, $P < 0.005$ and $62.6 \pm 1.2\%$; $t_8 = 10.25$, $P < 0.005$, respectively; Fig. 4). This shows that MAM treatment did not impair the ability of naïve rats to learn about the objects and to express short-term recognition memory. At the longer retention intervals, MAM-treated rats spent an equal amount of time exploring the novel and familiar objects (Fig. 4), indicating a similar decay of recognition memory as that observed in saline–naïve rats (Newman–Keuls, $P > 0.05$ at both 24 and 48 h delay).

The effect of reducing neurogenesis during enrichment on short- and long-term recognition memory was then assessed by comparing the performance of saline–enriched and MAM–enriched rats (Fig. 4). During the acquisition phase, the total exploration time did not differ between groups (65.7 ± 6 and 63.2 ± 6.2 s, respectively; Newman–Keuls, $P > 0.05$; data not shown). This indicates that animals expressed similar motivation and spontaneous attraction for exploring the objects, independent of the pharmacological treatment and environmental conditions. When tested 1 h after acquisition, saline–enriched and MAM–enriched groups exhibited a similar exploratory preference for the novel object ($65.4 \pm 3.9\%$; $t_{12} = 3.94$, $P < 0.005$ and $71.1 \pm 2.6\%$; $t_{10} = 6.68$, $P < 0.005$, respectively; Fig. 4). This confirmed that MAM treatment did not alter short-term recognition memory. However, when testing was conducted 24 or 48 h after the acquisition phase, enriched rats treated with MAM showed no preference for the novel object ($47.5 \pm 3.4\%$ and $51.5 \pm 3.6\%$ at 24 and 48 h; $P > 0.05$ at both delays; Fig. 4), in contrast to saline–enriched rats ($64.5 \pm 1.7\%$ and $62.1 \pm 1.3\%$, at both 24 and 48 h delay). The Newman–Keuls *post hoc* analysis showed a significant difference between these two groups at both delays ($P < 0.005$ and $P = 0.005$ at 24 and 48 h, respectively; Fig. 4). This impairment of recognition memory in MAM-treated rats at long delays despite enrichment was further confirmed using repeated-measures ANOVA ($F_{1,21} = 7.97$, $P = 0.01$). In fact, the overall performance of MAM-treated enriched rats was the same as that of the naïve controls ($F_{1,24} = 0.55$, $P > 0.05$; Fig. 4), suggesting that MAM treatment completely blocked the facilitating effect of enrichment on recognition memory. These results suggest that enrichment-induced neurogenesis is necessary for the facilitation of long-term, but not short-term, recognition memory after enrichment.

Discussion

Although adult neurogenesis has now been demonstrated in a number of species, the functional role of newborn neurons in cognitive functions such as learning and memory remains a matter of debate. The present study was designed to determine whether enrichment-induced enhancement of adult neurogenesis is required for the expression of memory improvement following environmental enrichment. Our experimental strategy was to use a behavioural enrichment paradigm to increase neurogenesis, to block proliferation and thus reduce the number of dividing cells using the antimitotic drug MAM, and then examine memory performance. We report that a short, daily exposure to an enriched environment in adult rats results in an increase in newly generated cells in the dentate gyrus associated with enhancement of long-term recognition memory, and that the reduction of neurogenesis during enrichment completely prevents the enhancement of long-term recognition memory.

In agreement with previous studies, we found that exposure to an enriched environment has a survival-promoting effect on newly generated cells in the dentate gyrus of rodents (Kempermann *et al.*, 1997, 1998b; Nilsson *et al.*, 1999; van Praag *et al.*, 1999b). Our data further demonstrate that a short (3 h) daily exposure to a complex environment for 14 days in adults is sufficient to induce a long-term increase in the rate of neurogenesis and a marked pro-mnemonic effect. Enrichment induced an $\approx 70\%$ increase in the number of newborn dentate gyrus cells and this was not associated with any detectable change in differentiation of the progenitor cells towards a neuronal or glial fate. Indeed, most (80–85%) of these cells expressed a neuronal phenotype, a proportion similar to that observed in naïve rats. This suggests that the survival-promoting effect of enrichment is expressed independently of the cell lineage (see also Nilsson *et al.*, 1999), resulting in a net increase in both neuronal and glial cells in the dentate gyrus, although proportionally most of these new cells are neurons.

In terms of memory performance, our results show that enrichment has a beneficial effect on recognition memory. Specifically, using a trial-unique object recognition task with a very brief exposure to objects that does not lead to long-term memory in rats housed in standard conditions, we found that enriched rats became able to form a long-term memory lasting at least 48 h. These results confirm and extend previous reports that enrichment facilitates learning in various spatial and nonspatial tasks (Kempermann *et al.*, 1997; Rampon *et al.*, 2000). Enrichment is a complex situation in which animals encounter stimuli of different sensory–motor modalities and indulge in social interaction (Rosenzweig, 1966). The critical elements in mediating learning facilitation in different types of tasks are difficult to identify and are probably diverse. In the present case, the marked permissive effect on long-term memory for objects might be related to the nature of the enrichment procedure which consisted of exposing rats to a variety of objects and toys each day, providing ample opportunity for the animals to improve their cognitive ability to memorize objects. Enrichment, however, was not associated with a greater tendency to explore the objects during acquisition, nor was it associated with an increased short-term memory performance. In all, these results suggest that in this task enrichment had a beneficial effect on memory consolidation.

Enrichment results in many different types of structural and functional changes that could contribute to memory facilitation, including increased dendritic branching and spine and synapse numbers in cortex and hippocampus (reviewed in van Praag *et al.*, 2000; Mohammed *et al.*, 2002). In addition to increased neurogenesis, enhanced synaptogenesis has been observed in the dentate gyrus and areas CA3 and CA1 (Altschuler, 1979; Rampon *et al.*, 2000). Here, using the cytostatic drug MAM to reduce neurogenesis throughout the enrichment period, we examined the contribution of neurogenesis to enrichment-induced memory facilitation. In naïve rats, MAM injections resulted in a substantial but not complete loss of BrdU-labelled cells in the dentate gyrus (see also Shors *et al.*, 2001). After

enrichment, a more profound depletion of BrdU-labelled cells was found, so that the residual neurogenesis level was similar in naïve and enriched rats. This may reflect a higher rate of progenitor cell proliferation at the time of MAM injections, suggesting that exposure to enriched environment may favour cell mitosis. Although our protocol was not designed to measure cell proliferation *per se*, this interpretation is consistent with reports of enhanced proliferation after enrichment in certain mice strains (Kempermann *et al.*, 1998a) although not in all strains (Kempermann *et al.*, 1997). Analysis of double-labelled cells revealed that MAM alone did not affect neuronal–glial differentiation. However, there was a tendency for an increase in the proportion of BrdU single-labelled cells in enriched MAM-treated rats. The reason for this is unknown. A slower differentiation rate in a pool of newborn cells that resisted MAM treatment, or a higher death rate in a pool of newly differentiated neurons during enrichment, might be contributing factors. Nonetheless, MAM treatment during enrichment caused a marked reduction in the number of newborn cells in the dentate gyrus at the time of memory testing, a very large proportion of these being neurons.

Results from the memory test demonstrate that the reduction in neurogenesis during enrichment completely prevented the memory-enhancing effect of enrichment. In MAM-treated enriched rats, a severe deficit in long-term recognition memory was observed compared to enriched, nontreated rats, with a level of performance no different from that of rats housed in standard conditions. The fact that MAM, in naïve or enriched rats, caused no change in object exploration time and that short-term memory was intact, rules out the possibility that the deficit at 24 and 48 h results from nonspecific effects such as impaired motor activity, reduced tendency to explore novelty (see also Shors *et al.*, 2002), or to an inability to learn about the objects. Thus, our findings demonstrate that neurogenesis contributes to the expression of memory enhancement induced by behavioural enrichment and they add support to the view that neurogenesis plays an important role in memory.

In our experiment we focused on neurogenesis in the dentate gyrus. It is likely, however, that MAM also reduced neurogenesis in the subventricular zone, with a consequential reduction in the number of newborn cells migrating to the olfactory bulb (Luskin, 1993; Rochefort *et al.*, 2002). Can this contribute to the behavioural effects observed here? Although we cannot totally exclude this possibility, we believe it unlikely as every step was taken to eliminate olfactory cues during the object recognition test. Moreover, Brown *et al.* (2003) recently showed that environmental enrichment stimulates hippocampal but not olfactory bulb neurogenesis. Thus, we conclude that the reduction in neurogenesis in the dentate gyrus is the prime cause for abolishing the memory-enhancing effect of enrichment.

One issue that is currently debated is the contribution of the hippocampal formation to object recognition memory in rodents. While it has been reported that recognition memory at relatively short delays (4 h) is spared by hippocampal lesions (see Mumby, 2001; for a review), other studies have found a delay-dependent impairment in long-term (24 h) recognition memory in rats with hippocampal lesions (Vnek & Rothblat, 1996; Clark *et al.*, 2000; but see Gaskin *et al.*, 2003) and mice with lidocaine-induced inactivation of the hippocampus (Hammond *et al.*, 2004). Two recent studies have unequivocally shown that the impairment depends on lesion size in rats (Broadbent *et al.*, 2004) and that post-training lesions of the hippocampus are associated with retrograde, although not anterograde, amnesia in recognition memory, suggesting that the hippocampus, when functional, participates in the formation of recognition memory (Gaskin *et al.*, 2003). In addition, pharmacological and genetic studies have shown that intrahippocampal administration of the NMDA receptor

antagonist APV (Baker & Kim, 2002), and hippocampal-specific NMDA receptor NR1 (Rampon *et al.*, 2000) or CREB (Pittenger *et al.*, 2002) gene inactivation in mutant mice, impair long-term object recognition memory. In corroboration, higher levels of NMDA NR1 subunit expression in the hippocampus correlates with object recognition performance (Xu *et al.*, 2003) and hyperphosphorylation of MAP kinase is observed in the dentate gyrus and hippocampus proper after exploration of objects and inhibiting MAP kinase phosphorylation impairs long-term but not short-term memory (Kelly *et al.*, 2003). In all, these studies lend strong support to the conclusion that the hippocampus is important for object recognition memory at least under certain circumstances. Based on these data, the most parsimonious account would suggest that hippocampal involvement in object recognition increases with task demands (e.g. identical vs. different objects during training), the size and shape of the testing apparatus providing more local or distant contextual information (see Winters *et al.*, 2004), the ability to form spatial configurations and associative relationships between items (Jenkins *et al.*, 2004) and the retention delay (Hammond *et al.*, 2004). In our experiment, we specifically devised a procedure that would most probably increase the demand on the hippocampus by using a wide open-field which provides contextual information, two different objects during the sample phase, and long retention delays. Under these experimental conditions, we report that increasing neurogenesis by enrichment facilitates long-term but not short-term recognition memory in rats. This result, in line with the current literature, suggests that at least under these experimental conditions the hippocampal formation contributes to long-term object recognition memory.

Several experiments have highlighted the potential contribution of newborn neurons to cognitive processes. For example, forms of hippocampal-dependent learning enhance hippocampal neurogenesis (Gould *et al.*, 1999a) whereas decreasing the number of newborn granule cells impairs trace conditioning, although not disrupting the formation of spatial and contextual fear memories (Shors *et al.*, 2001, 2002). In this experiment we did not address the issue of the effect of MAM on recognition memory because we purposely used a protocol that does not lead to long-term memory in naïve rats, but we did find that MAM does not affect short-term recognition memory, confirming that short-term recognition memory does not require neurogenesis (Madsen *et al.*, 2003; Raber *et al.*, 2004). Related observations include the correlation between hippocampal neurogenesis and acquisition of new information observed in inbred strains of mice (Kempermann & Gage, 2002) or aged rats with differential learning performance (Drapeau *et al.*, 2003), or the finding that increased neurogenesis produced by physical exercise improves spatial learning (van Praag *et al.*, 1999a). Conversely, reduction of neurogenesis associated with early isolation rearing (Lu *et al.*, 2003) or prenatal stress (Lemaire *et al.*, 2000) was shown to lead to learning deficits. In the context of these initial studies, our results suggest that new dentate granule cells generated during enrichment are critically involved in enrichment-induced facilitation of memory consolidation. Functionally, these findings support one current view that newborn cells may increase the ability for future learning by providing more cells available for recruitment into existing circuits (for reviews see Gould *et al.*, 1999b; Gross, 2000; Kempermann *et al.*, 2004; Shors, 2004). Within a few weeks newborn granule cells become functionally integrated into hippocampal circuitry (Hastings & Gould, 1999; Markakis & Gage, 1999) and develop physiological characteristics of mature granule cells (van Praag *et al.*, 2002). Evidence also shows that they can express a learning-induced immediate–early gene response (Jessberger & Kempermann, 2003). Clearly, the mechanisms mediating the neurogenesis-promoting effect of enrichment and how new

neurons provide conditions favouring the formation of memories are not yet known. One testable hypothesis is that an increase in the number of functional mature neurons and of immature neurons known to exhibit unique membrane properties (Wang *et al.*, 2000; Snyder *et al.*, 2001; Schmidt-Hieber *et al.*, 2004) may increase the capacity for rapid structural and functional changes at the synaptic and dendritic levels, thereby increasing the efficiency of a neural system for encoding and/or consolidating newly acquired information.

Acknowledgements

Abbreviations

BrdU, 5-bromo-2′-deoxyuridine; GFAP, glial fibrillary acidic protein; MAM, methylazoxymethanol acetate; PB, phosphate buffer; PBST, phosphate buffer containing 0.25% Triton X-100; Tuj-1, class III β-tubulin.

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