

Short-term survival of newborn neurons in the adult olfactory bulb after exposure to a complex odor environment

Christelle Rochefort and Pierre-Marie Lledo

Pasteur Institute, Laboratory of Perception and Memory, CNRS UMR 2182, 25 rue du Dr Roux, 75015 Paris, France

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Abstract

In the olfactory bulb of adult mice, new neurons are continually integrated into existing neuronal networks. Previous studies have demonstrated that exposure to a complex odor environment increases the incorporation of newborn bulbar neurons without modifying the proliferation rate. Whether this incorporation is transient or leads to the long-lasting presence of new neurons has not yet been answered. Because a transient increase of new neurons impacts olfactory information processing differently than a long-lasting increase, we conducted experiments to investigate the time course of survival and cell death of newly generated bulbar neurons following exposure to an enriched olfactory environment. Dividing cells were labeled with bromodeoxyuridine (BrdU) and were counted at several survival time points thereafter. Interestingly, whereas the number of surviving BrdU-labeled cells was elevated at the time when animals were withdrawn from their enriched housing, this number returned to control level 1 month later. Similarly, when olfactory memory was investigated, we found that the improvement of short-term memory, induced by enriched odor exposure, lasted less than 1 month. These findings indicate not only that the recruitment of newborn neurons closely followed the degree of environment complexity, but also that olfactory memory is tightly associated with the level of ongoing neurogenesis in the adult olfactory bulb.

Introduction

Although it is becoming clear that adult neurogenesis represents an important mode of structural modification of the olfactory bulb and the dentate gyrus of the hippocampus, its functional significance remains highly debated. It has been reported that the presence of newborn neurons in the adult hippocampus may be associated with the formation of new trace memories (Shors *et al.*, 2001). By contrast, other studies have proposed that hippocampal neurogenesis might rather represent a clearance of the outdated memory traces following consolidation, thus ensuring that this structure is again available to process new memories (Feng *et al.*, 2001), and that this phenomenon could occur specifically during the late phase of hippocampal learning (Döbrösy *et al.*, 2003). Whatever the precise function of neurogenesis, there is a converging view according to which both production and survival of newborn neurons in the adult forebrain (i.e. the hippocampus and the olfactory bulb) are tightly regulated by the level of experience (Corotto *et al.*, 1994; Kempermann *et al.*, 1997; van Praag *et al.*, 1999; Rochefort *et al.*, 2002) and humoral factors (reviewed in Gould *et al.*, 2000; Abrous *et al.*, 2005). This indicates that adult-generated neurons can be used as a substrate by which experience or internal state influence normal brain functions. Therefore, adult neurogenesis might be seen as a neuroadaptive response to

various kinds of challenges (reviewed in Abrous *et al.*, 2005; Lledo *et al.*, 2005) but whether it is a transient or a long-lasting adaptation still remains unknown.

In the olfactory bulb, new neurons are continuously added to different layers to integrate into pre-existing networks (Luskin, 1993; Lois & Alvarez-Buylla, 1994). This constitutive neurogenesis is regulated in response to functional challenges, including enriched odor environments (Rochefort *et al.*, 2002). Although the new neurons acquire the electrophysiological properties of bulbar interneurons (Belluzzi *et al.*, 2003; Carleton *et al.*, 2003), questions remain as to whether the new neurons participate in the bulbar circuits, if so whether they contribute in a persistent or transitory manner, and whether memory performance remains tightly associated with the number of newborn neurons recruited into the adult olfactory bulb.

It is well known that an enriched environment (e.g. complex sensorial stimulations, physical exercise or social experiences) modifies brain functioning and its anatomical organization (e.g. Bennett *et al.*, 1969; Turner & Greenough, 1985; Nakamura *et al.*, 1999; reviewed in Abrous *et al.*, 2005). Most of these responses may occur at early stages, before maturation of the developing brain is achieved. However, the continuous production of neurons during adulthood still allows some brain areas to adjust their functioning to these challenges throughout life. Hence, adult neurogenesis might permit the mature neuronal network to respond to functional demands.

As newborn neurons are structurally plastic (Schmidt-Hieber *et al.*, 2004) and therefore highly susceptible to changes according to life experiences, the question of the persistence, or not, of new neurons becomes particularly relevant. The current study aims to analyse the time course of newborn cell survival in the adult olfactory bulb

Correspondence: Dr C. Rochefort, at *present address below, and Dr P.-M. Lledo, as above.

E-mails: rochefort@molgen.mpg.de; pmlledo@pasteur.fr

*Present address: Max-Planck Institute for Molecular Genetics, Ihnestr. 73, 14195 Berlin, Germany.

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following odor enrichment, and to investigate whether changes in bulbar neurogenesis are associated with modifications of olfactory performance. We show that adult neurogenesis and odor memory are together increased following a period of odor enrichment, but rapidly return to basal levels once animals are replaced to standard housing conditions for a period of 30 days. These results demonstrate that the constant integration of newborn neurons into the functional bulbar circuit closely follows the degree of environmental complexity.

Methods

Housing conditions and animals

Male C57BL/6J mice (2 months old) were bred in our animal facilities. Animals were randomly assigned to three experimental groups. The enriched group consisted of animals housed in an odor-exposure environment for 40 days. Enriched mice were exposed daily for 24 h to different aromatic fragrances that were placed in a tea ball hanging from the cover of standard breeding cages. The two other groups consisted of enriched animals that returned to standard housing for 30 days (Enr-std30) or 60 days (Enr-std60), following enrichment

(Fig. 1a and b). For each experimental condition, standard mice were reared under the same conditions except that the tea ball was left empty. Three days before the behavioral experiments, mice were housed singly in polycarbonate cages (32 × 14 × 20 cm). Behavioral experiments took place in the home cage of the tested animals during the dark phase of the day–night cycle, under a red light. Animals were allowed access to food and tap water *ad libitum* and were kept on a reversed light–dark cycle (lights off from 11:00 to 23:00 h). Animals used for the immunohistochemistry and the behavioral analyses come from different groups. Throughout all experiments, the observer was blind to the experimental conditions. All experimental procedures were in accordance with the Society for Neuroscience and European Union Guidelines, and were approved by our Institutional Animal Care and Utilization Committees.

BrdU labeling and detection

Newborn cells were labeled with 5-bromo-2'-deoxyuridine (BrdU, Sigma), a marker of DNA synthesis (50 mg/kg, i.p.). Following a 20-day period of enrichment, four injections repeated every 2 h were administered to animals. Labeled cells were detected after three

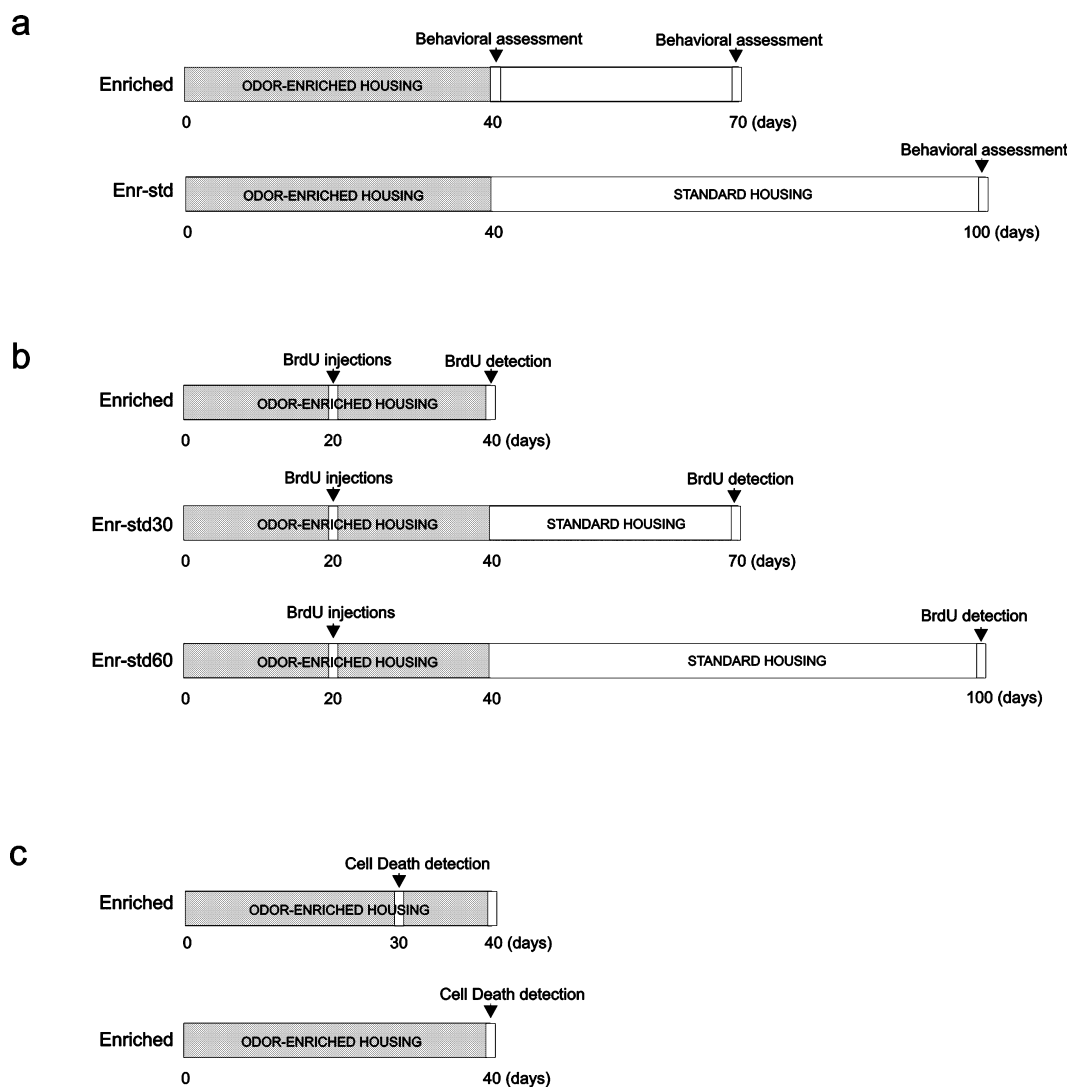


FIG. 1. Timetable of experimental design. Protocol a was used to assess olfactory memory after different times of standard housing subsequent to enrichment. Protocol b was used to assess the survival of neurons born during the enrichment phase after the return to standard housing for different periods of time. Protocol c was used to assess cell death using TUNEL after 30 or 40 days following the beginning of enrichment. Standard mice of each group were held under standard laboratory housing conditions.

different survival times (Fig. 1b). The mice of the enriched group were replaced in their respective cages for a further 20 days until the end of the period of olfactory enrichment. The mice of the Enr-std30 or Enr-std60 groups were kept in standard housing for, respectively, 30 or 60 days after enrichment.

Following the housing period, mice were given an overdose of sodium pentobarbital (100 mg/kg; Sanofi, France) and perfused transcardially with 50 mL of saline (NaCl 0.9%) containing heparin (5×10^3 units/mL) at 37 °C followed by 200 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3). The brain was excised and immersed overnight in the same fixative at 4 °C. Coronal sections were serially cut using a vibrating microtome (Leica) and collected in PBS (0.1 M; pH 7.3). BrdU staining was performed in a one-in-three series of free-floating 40- μ m sections (120 μ m apart) that were pretreated for DNA denaturation (2 N HCl for 30 min at 37 °C). The primary antibody used was a rat monoclonal anti-BrdU antibody (1 : 200; ImmunologicalsDirect, UK). The number of BrdU-labeled cells was determined by peroxidase revelation (ABC system, Vector Laboratories, Inc., Burlingame, CA, USA) with biotinylated donkey anti-rat IgG antibodies (1 : 200, Vector Laboratories) and diaminobenzidine (0.05%) as the chromogen (Sigma).

Detection of apoptosis

Apoptotic cell death was detected, 30 and 40 days after starting enrichment (Fig. 1c), using the TUNEL staining method (terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling, Apoptag kit, Intergen Co.). Immunohistochemistry was carried out on 8- μ m-thick coronal sections of olfactory bulb to detect DNA fragmentation *in situ*. After deparaffinization and rehydration, the tissue was treated with 0.5% Triton X-100 for 10 min and then incubated for 15 s in equilibrium buffer (Serological Corp.). Sections were then incubated for 1 h in a humidified chamber at 37 °C with a solution containing terminal deoxynucleotidyl transferase and digoxigenin nucleotides (Serological Corp.). After vigorous washing, a peroxidase-labeled anti-digoxigenin antibody (Serological Corp.) was added for 30 min at room temperature, and staining was revealed by diaminobenzidine.

Quantification

To assess the density of new cells in the olfactory bulb, BrdU-stained nuclei, observed with a 20 \times objective (Olympus), were counted within the entire granule cell area. The numbers of positive profiles were then related to the granule cell layer sectional volume to obtain a density per mm³. To count TUNEL⁺ cells in the granule cell layer and in the rostral migratory stream of the olfactory bulb (RMS_{OB}), these areas were delineated manually on sections counterstained with Methyl Green (Vector Laboratories).

Behavioral experiments

To explore olfactory memory, 3 days before tests were run, animals were exposed to odor stimuli in their home cage, different from those used in the test session, to familiarize them with the procedure. A test session consisted of two 5-min presentations of the same odor with a 30-, 120- or 180-min inter-presentation interval. These different intervals were tested randomly in separate sessions spaced at least 24 h apart. Different odors (octanol, hexanol, amyl acetate, carvone, limonene, propionaldehyde, heptanol, caprinaldehyde, butyraldehyde and propanol) were used in each test and counterbalanced across all

the different delay conditions. We recorded the time spent by the animals rearing and sniffing at the odor. A significant decrease in investigation time upon second presentation of an odor indicates retention of that odor. For the retroactive interference test, mice were presented with a different odor (odor-2) 5 min after having been exposed to the first (odor-1). This was followed by a second presentation of odor-1, 30 min after its first presentation.

Statistics

Behavioral data were compared between experimental and standard groups using a Wilcoxon's test, and anatomical data using a Student's *t*-tests. Levels of significance were set at $P < 0.05$.

Results

Odor-enriched housing and olfactory performance

During the first 5 min of odor presentation, enriched and standard mice spent the same amount of time exploring one novel odor (first presentation of the 30-min interval test, $P > 0.05$; first presentation of the 120-min interval test, $P > 0.05$; first presentation of the 180-min interval test, $P > 0.05$) (Fig. 2a and b). These data indicated that both groups have equivalent levels of motivation and curiosity. However, mice housed in an odor-enriched environment for 40 days improved their performance on a short-term memory test (Fig. 2a and b). They showed a significant decrease in odor investigation during the second exposure at 30, 120 and 180 min ($P < 0.05$, $P < 0.001$, $P < 0.05$, respectively) (Fig. 2b). By contrast, standard mice were able to retain a trace for short inter-trial intervals (e.g. 30 and 120 min) but not for 180 min ($P < 0.01$, $P < 0.01$, $P > 0.05$, respectively) (Fig. 2a).

In a retroactive interference test, we analysed the ability to recognize an odor (odor-1) that was followed by exposure to a different interfering odor (odor-2) after the first presentation (Fig. 2c and d). Odor-1 was presented 30 min after its initial presentation, at a time when the two groups recognize a familiar odor. Enriched mice spent less time investigating odor-1 during the second presentation despite the exposure to odor-2 ($P < 0.001$) (Fig. 2d), demonstrating that they recognized the first odor and that the immediate presentation of an interfering odor did not preclude the storage and the recall of the first odor memory. By contrast, the investigation time of standard animals for the second presentation of odor-1 did not significantly differ from the initial presentation ($P > 0.05$) (Fig. 2c). This means that exposure to a distracting odor immediately following the presentation of a first one interfered with subsequent recognition of the latter in standard mice only.

Odor-enriched stimuli withdrawal and olfactory performance

The Enr-std group consisted of animals living in an enriched environment for 40 days before returning to standard housing for 2 months (Fig. 1a). This group was tested for both odor recognition and retroactive interference 2 months after the end of enrichment. Interestingly, the performance of the Enr-std group was not different from those of standard animals. Both were able to recognize odors when the interval of time tested was 30 or 120 min (standard, $P < 0.01$ and $P < 0.01$; Enr-std, $P < 0.01$ and $P < 0.01$) but not for 180 min (standard and Enr-std, $P > 0.05$) (Fig. 2e and f). Similarly, in the retroactive interference test, Enr-std and standard mice showed comparable performance: both groups were unable to recognize odor-1 after 30 min (standard, and Enr-std, $P > 0.05$) (Fig. 2g and h).

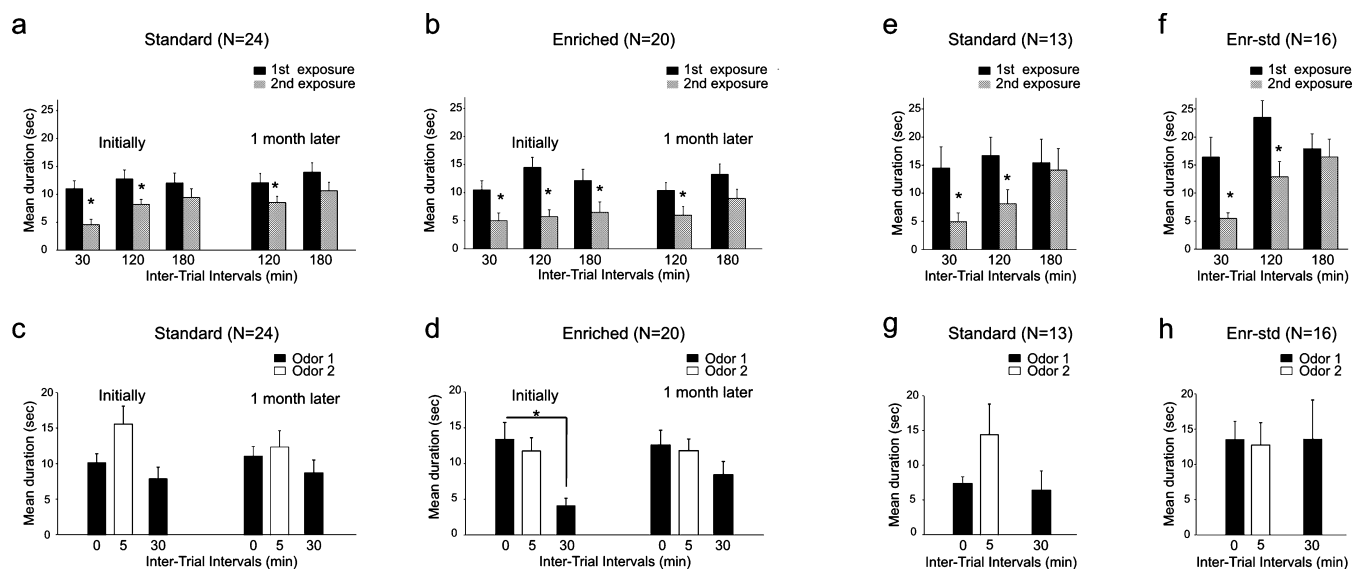


FIG. 2. The withdrawal of the enriched environment induced the return of olfactory performance to control levels. (a, b, e and f) Effect of different inter-trial intervals on odor recognition in standard and enriched mice. Each bar represents the mean time investigating a given odor on the first exposure (black column) and on the second exposure (grey column) in standard (a and e), enriched (b) and Enr-std (f) animals. (c, d, g and h) Effect of the presentation of a novel odor (white column, odor-2) 5 min after the presentation of the first odor (black column, odor-1) on subsequent odor-1 recognition. * $P < 0.05$ with a Wilcoxon's test.

To ensure that the decrease in investigation time observed at different time intervals was specific to familiar odors and therefore reflected memory rather than a non-specific process, the specificity of odor recognition was assessed. Mice were first exposed to an odor and then, following a particular interval of time, were exposed to a second different odor. For the two groups of animals and at all time intervals tested, the exploration times were not significantly different (data not shown).

As the consequences on olfactory memory were not detectable 2 months after the end of enrichment, we investigated a shorter delay. One month after the end of enrichment, the same animals that showed better performance than standard animals at the end of enrichment were again tested for both odor recognition and retroactive interference tests (Fig. 2a–d). Again, no detectable differences were observed between the two groups. Both enriched and standard mice showed a significant reduction in investigation time for the 120-min interval (standard, $P < 0.05$; enriched, $P < 0.05$) but not for the 180-min interval (standard, $P > 0.05$; enriched, $P > 0.05$). During the retroactive interference test, neither of the two groups was able to recognize odor-1 after 30 min (standard, $P > 0.05$; enriched, $P > 0.05$). Therefore, regardless of the protocol used (i.e. consecutive memory tests with 1 month delay between each test, or animals tested for the first time 2 months after enrichment), the results were similar: environmental enrichment with subsequent stimulus withdrawal resulted in the return of increased olfactory performance to control levels.

Effects on bulbar neurogenesis

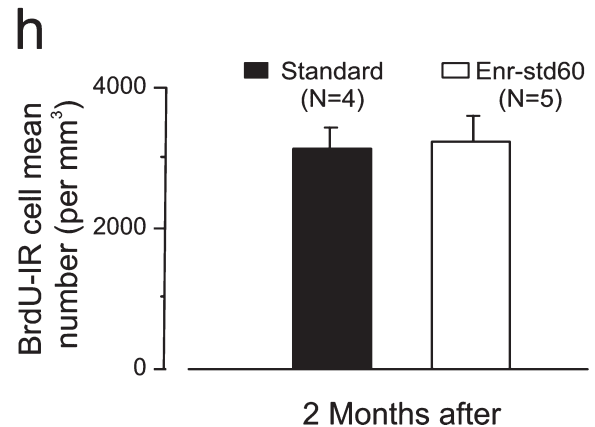
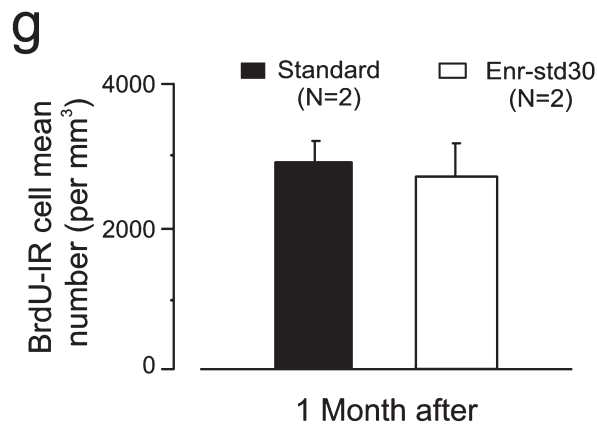
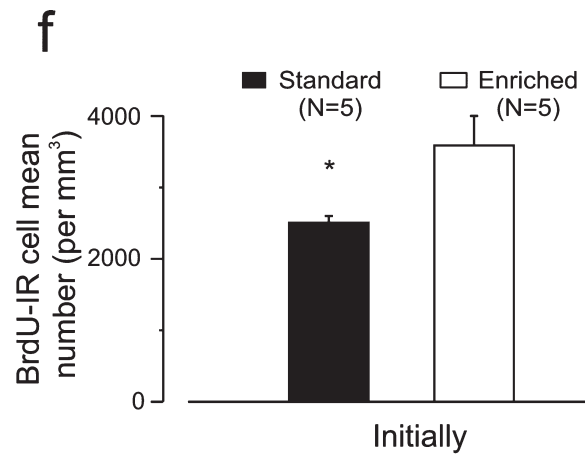
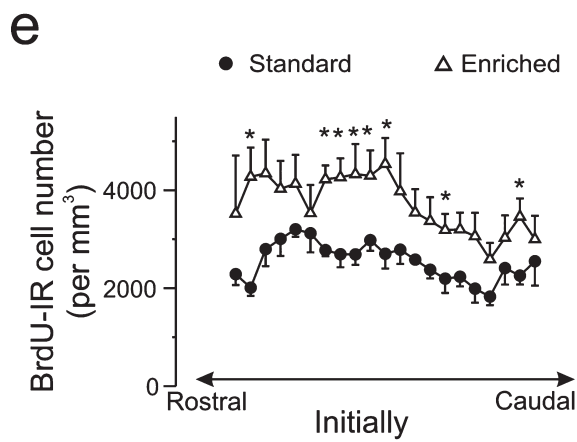
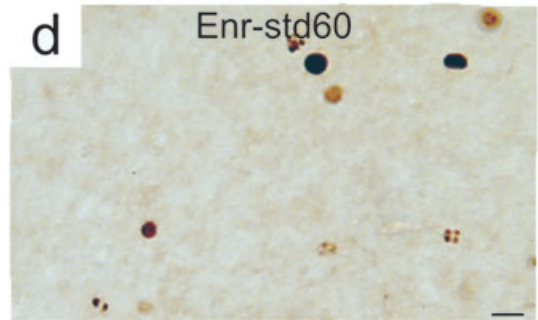
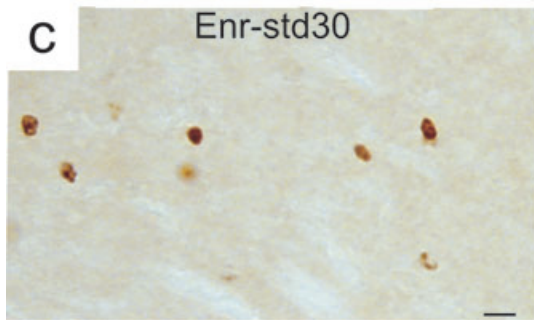
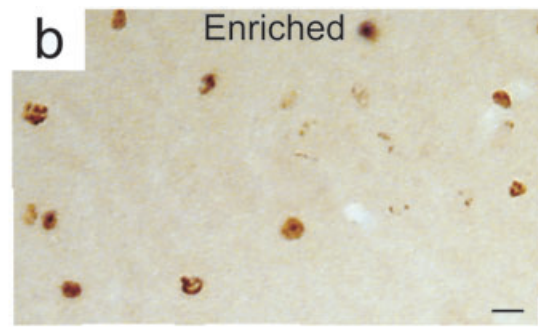
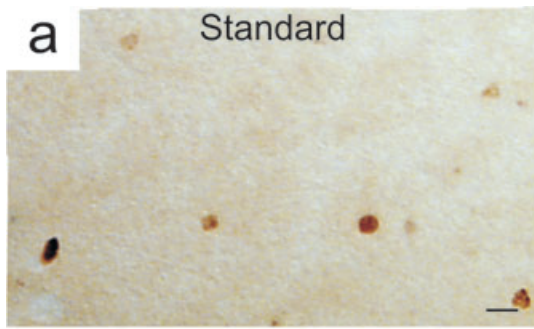
We assessed the rate of newborn granule cell recruitment in animals housed in an odor-enriched environment for 40 days and in enriched

animals that returned to standard housing for 1 or 2 months after the period of enrichment (Enr-std30 and Enr-std60). As reported previously (Rochefort *et al.*, 2002), the enrichment resulted in a 50% increase in the number of newborn neurons in the olfactory bulb ($P < 0.05$) (Fig. 3a, b, e and f). The density of BrdU⁺ cells was always higher in enriched mice than in standard mice throughout the entire rostro-caudal axis of the olfactory bulb (Fig. 3e). However, 1 or 2 months after enrichment, the number of new neurons did not differ between standard and enriched animals (standard vs. Enr-std30 groups, $P > 0.05$; standard vs. Enr-std60 groups, $P > 0.05$) (Fig. 3c, d, g and h). The neuronal phenotype of newborn cells was confirmed with co-labeling BrdU⁺ cells with the neuronal marker, NeuN (Rochefort *et al.*, 2002; data not shown). Together, these data clearly showed that enrichment has no long-lasting recruitment-promoting effect on newly generated bulbar interneurons.

Effect on cellular proliferation

We showed previously that enrichment did not affect the rate of proliferation in the subventricular zone of the lateral ventricles (Rochefort *et al.*, 2002). As it has been demonstrated that proliferation of precursors destined to the olfactory bulb occurs also in the RMS_{OB} (Gritti *et al.*, 2002), we investigated whether enrichment might have an effect on cell proliferation in the RMS_{OB}, an area closer to sensory inputs. We quantified the rate of newborn cells in the RMS_{OB} 4 h after a single BrdU injection. Again, we did not find any difference between standard and enriched animals (data not shown, $n = 9$; $P > 0.05$). This suggests that the temporary increase in newborn neuron recruitment results from longer survival, or a facilitated radial migration, rather than a higher proliferation rate in the olfactory bulb of enriched animals.

FIG. 3. The recruitment of newborn cells in the olfactory bulb is temporarily increased by odor exposure. (a–d) Representative photomicrographs of coronal sections through the olfactory bulb granule cell layer showing BrdU-stained cells at different times after the odor exposure in standard (a), enriched (b) and Enr-std (c and d) mice. Scale bars, 10 μ m. (e) Total number of BrdU-stained cells per mm³ throughout the rostro-caudal level axis of the olfactory bulb directly after odor exposure. (f–h) Mean number of BrdU-stained cells per mm³ for each group at different times after enrichment. * $P < 0.05$ with a Student's *t*-test.



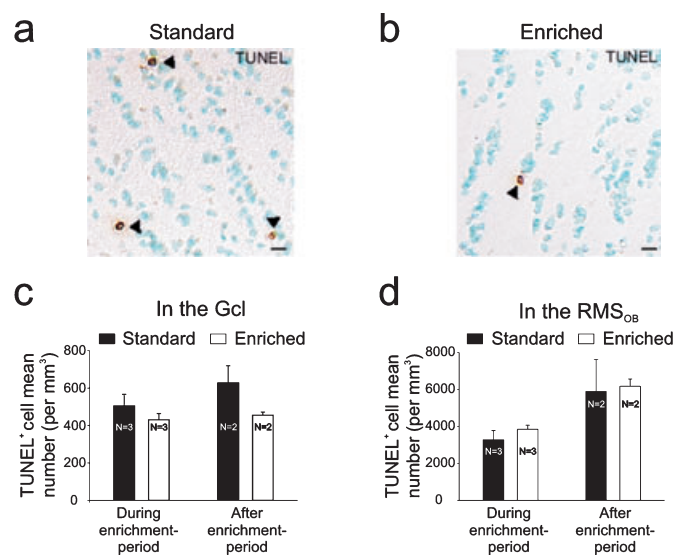


FIG. 4. Apoptotic cell death with nuclear fragmentation was detected throughout the adult olfactory bulb using TUNEL. (a and b) Representative sections through the granule cell layer of a standard- (a) and an enriched-caged (b) mouse, showing TUNEL⁺ cells. The sections were counterstained with methyl green. Scale bars, 10 μ m. (c and d) Mean number of TUNEL⁺ cells per mm³ for each group in the Gcl (c) and in the RMSOB (d), during and at the end of enriched housing. Gcl, granule cells layer; RMSOB, rostral migratory stream of the olfactory bulb.

Cell death quantification

To determine whether the elimination of granule cells might be sensitive to enrichment, we analysed programmed cell death using TUNEL after 30 or 40 days of enrichment (Fig. 4a and b). Previous studies have reported that neuronal progenitors are, in their vast majority, eliminated in the RMS (Morshead & Van der Kooy, 1992). Our stereological quantification confirmed that the density of apoptotic cells in the granule cell layer is lower than that measured in the RMS (standard group, 505 ± 62 vs. 3281 ± 495 cells/mm³, respectively; $P < 0.05$) (Fig. 4c and d). However, although the mean number of apoptotic granule cells in the enriched group was $\sim 30\%$ lower than in the standard group, no statistical difference was found ($P > 0.05$).

Discussion

Transient existence of adult-generated neurons in the olfactory bulb

As previously reported (Rochefort *et al.*, 2002), we found that olfactory enrichment (i.e. relatively less deprived than usual laboratory housing) dramatically increased bulbar neurogenesis and concomitantly improved olfactory memory in adult mice. We investigated how long these effects could last once enrichment ceased. We found that the number of newborn bulbar neurons returned to control levels as early as 1 month after animals were withdrawn from the enriched environment. In agreement with this, a previous study reported that adult mice living in an enriched environment and analysed for hippocampal neurogenesis 3 months later presented a degree of neurogenesis similar to mice living in standard conditions (Kempermann & Gage, 1999). Several other reports have already pointed out that experience-induced brain structural alterations are reversed following enrichment withdrawal (e.g. Bennett *et al.*, 1974; Green & Greenough, 1986). In the olfactory bulb, the effects of naris occlusion on both precursor cell proliferation and survival are reversed

following naris re-opening (Cummings *et al.*, 1997). In line with this, our observations point toward a reversible activity-dependent regulation of adult neurogenesis.

Survival of newborn neurons

Our second goal was to assess the contribution of apoptosis to the regulation of activity-dependent neurogenesis in the adult olfactory bulb. We did not find any significant difference in the rate of apoptosis between enriched and standard animals, although enriched animals tended to show a lower density of apoptotic cells. One explanation of this result could be that odor enrichment principally facilitates neuroblast recruitment (i.e. through an increase in tangential or radial migration), without changing the rate of cell death. This is consistent with the finding that expression of tenascin-R in the olfactory bulb depends on the degree of sensory input and that tenascin-R facilitates the incorporation of newborn neurons into the bulbar circuit (Saghatelian *et al.*, 2004). However, according to *in silico* studies, changing olfactory environment does increase the survival of newly generated cells without modifying the total number of granule cells (Cecchi *et al.*, 2001). Data from songbird neurogenesis provide evidence that new neurons are recruited to replace dying older ones (Scharff *et al.*, 2000). Although the relationship between the birth of new neurons and the death of older ones is still unknown in mammals, some studies have demonstrated that cell death turnover occurs preferentially in young neurons (until 3 months after birth), challenging the hypothesis of a neuronal replacement (Petreanu & Alvarez-Buylla, 2002; Winner *et al.*, 2002).

An alternative hypothesis to explain our result is that decreased cell death of newly generated cells after enrichment is counterbalanced by an increase of cell death of older immature neurons (i.e. neurons born before the BrdU injections) maintaining the total rate of cell death, and thus the total population of granule cells, constant. More precise studies labeling apoptotic processes in combination with immature and mature neuronal markers, as well as a precise time-course analysis of newborn cell number in the RMS following marker injection, are needed to elucidate this key issue.

Function of adult neurogenesis

In parallel with activity-dependent variations of neurogenesis, we showed that adult enriched mice had improved olfactory performance if tested immediately after enrichment, but showed similar performance to standard mice when tested 1 month later. Thus, the maintenance of improved olfactory capacity seems, like the degree of neurogenesis, to require continuous environmental stimulation. This differs from recent studies performed on hippocampus that showed that long-term enrichment continues to have an effect on hippocampal learning (for a review see Kempermann, 2002) and that these effects are stronger after long-term stimulation than following a shorter one (Kobayashi *et al.*, 2002).

After enrichment, we observed a temporal association between the return of both neurogenesis and olfactory performance to the levels of standard animals. This result supports several reports showing striking parallels between levels of neurogenesis and learning and memory performance. It has been shown that neural cell adhesion molecule (NCAM) -mutant mice, which have reduced bulbar neurogenesis, displayed impaired olfactory discrimination (Gheusi *et al.*, 2000). In aging mice a decrease in bulbar neurogenesis, without a reduction in the total number of neurons, is also associated with impaired olfactory

discrimination (Enwere *et al.*, 2004). Similarly, a substantial reduction of hippocampal neurogenesis induced by anti-mitotic drug treatment impairs hippocampal memory, while the recovery of cell production is associated with the ability to acquire new trace memories (Shors *et al.*, 2001). Together, these studies indicate that adult-generated neurons are potentially involved in memory formation. Conversely, in a recent report, we have shown that neurogenesis and memory performances could be dissociated. Nicotinic acetylcholine receptor knockout mice have impaired olfactory memory despite an increase in bulbar neurogenesis (Mechawar *et al.*, 2004). However, although this study showed that enhanced bulbar neurogenesis is not sufficient to improve olfactory capabilities when cholinergic inputs are altered, it certainly did not rule out a role for neuronal replacement in memory performance.

It has been suggested that the activity-dependent regulation of hippocampal neurogenesis could be mediated by an excitation–neurogenesis coupling (Deisseroth *et al.*, 2004). In the olfactory bulb, too, an enriched olfactory environment may induce increased stimulation of the bulbar circuitry, leading to better survival of newborn neurons. Furthermore, young hippocampal neurons express facilitated synaptic plasticity (Schmidt-Hieber *et al.*, 2004) and such synaptic plasticity is enhanced by exposure to a novel environment (Davis *et al.*, 2004). If a similar situation pertains with regard to newborn neurons in the adult olfactory bulb, their abilities to undergo rapid structural change might allow these cells to be the best neuronal population to create associations between stimuli, forming a short-term olfactory memory trace in the bulb before that trace is transferred elsewhere for long-term storage.

This study provides the first quantitative data demonstrating that new neurons are inserted into the neuronal network of the olfactory bulb in a reversible manner, perhaps optimizing the bulbar network for the complexity of the sensory environment encountered. We propose that the transient nature of the adult-generated neurons of the olfactory bulb make them appropriate to play a role in some of the processes involved in memory formation. Ultimately, an analysis of neurogenesis occurring in ethologically relevant behavioral conditions is needed to unveil the precise relationships between adult neurogenesis and cognitive functions.

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Abbreviations

BrdU, 5-bromo-2'-deoxyuridine; RMS, rostral migratory stream; TUNEL, terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling.

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