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YOUNG HIPPOCAMPAL NEURONS ARE CRITICAL FOR RECENT AND REMOTE SPATIAL MEMORY IN ADULT MICE

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Abstract—New granule cells are continuously generated throughout adulthood in the mammalian hippocampus. These newly generated neurons become functionally integrated into existing hippocampal neuronal networks, such as those that support retrieval of remote spatial memory. Here, we sought to examine whether the contribution of newly born neurons depends on the type of learning and memory task in mice. To do so, we reduced neurogenesis with a cytostatic agent and examined whether depletion of young hippocampal neurons affects learning and/or memory in two hippocampal-dependent tasks (spatial navigation in the Morris water maze and object location test) and two hippocampal-independent tasks (cued navigation in the Morris water maze and novel object recognition). Double immunohistofluorescent labeling of the birth dating marker 5-bromo-2'deoxyuridine (BrdU) together with NeuN, a neuron specific marker, was employed to quantify reduction of hippocampal neurogenesis. We found that depletion of young adult-generated neurons alters recent and remote memory in spatial tasks but spares non-spatial tasks. Our findings provide additional evidence that generation of new cells in the adult brain is crucial for hippocampal-dependent cognitive functions.

Key words: neurogenesis, BrdU, dentate gyrus, MAM, spatial memory.

In the adult mammalian brain, new cells continue to be generated throughout adulthood in the dentate gyrus of the hippocampus. Several lines of evidence have shown that a substantial fraction of these newly born cells survive and become fully functional neurons (Ramirez-Amaya et al., 2006; Kee et al., 2007; Tashiro et al., 2007; Trouche et al., 2009). Each new granule cell undergoes a maturation process lasting several months. Ultimately, new neurons develop electrical properties that are highly similar to developmentally born granule cell and form synaptic connections with the same afferent and efferent neurons as mature granule cells (Song et al., 2002; van Praag et al., 2002; Esposito et al., 2005; Toni et al., 2008; Zhao et al., 2008). However, their maturation process progresses through states that make immature neurons distinct from mature granule cells. For example, during the first week after their birth, adult-generated neurons, although not yet synaptically connected to the hippocampal networks, receive a tonic activation by ambient GABA (Esposito et al., 2005; Ge et al., 2006; Zhao et al., 2006). During the second week after birth, adult-generated neurons extend their axon and dendrites and exhibit enhanced excitability compared to older granule cells (Wang et al., 2000; Snyder et al., 2001; Esposito et al., 2005) and appear particularly sensitive to modifications by experience (Gould et al., 1999; Leuner et al., 2004, 2006; Epp et al., 2007; Kee et al., 2007; Tashiro et al., 2007; Trouche et al., 2009; Tronel et al., 2010).

In line with this idea, we found that new granule neurons are recruited into neuronal networks that support retrieval of remote spatial memory (Trouche et al., 2009). We further reported that as initial training occurs, young neurons reaching the critical period of their maturation are predominantly recruited in the event a similar experience is encountered (Trouche et al., 2009). Based on these findings, we hypothesized that a loss of young neurons, at the time when they are very sensitive to surrounding neuronal activity, should specifically alter hippocampal function. To evaluate this hypothesis, we used the antimitotic agent methylazoxymethanol acetate (MAM) (Johnston and Coyle, 1979) to reduce numbers of young granule neurons prior to behavioral training. We then examined the effects of new neurons depletion on learning and memory using behavioral tasks which differed in their dependence on hippocampal function. Using two tasks that require an intact hippocampus (spatial navigation in the Morris water maze and object location) and two hippocampal-independent tasks (cue navigation in the Morris water maze and novel object recognition), we revealed the critical need for young neurons in the retrieval of recent and remote hippocampal-dependent spatial memory. This study further extends our knowledge on the role played by adult-generated hippocampal neurons in spatial memory processes, as a function of their age and learning context.

EXPERIMENTAL PROCEDURES

Animals and housing conditions

Adult male C57BL/6 mice (9 week-old, Charles River Laboratories, Orléans, France) were housed in fours in a temperature-

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Abbreviations: BrdU, 5-bromo-2'deoxyuridine; DG, dentate gyrus; MAM, methylazoxymethanol acetate; NeuN, neuron-specific nuclear protein.

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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 and the French National Committee (86/609/EEC).

BrdU injections and MAM treatment

We first identified a dose for MAM administration in mice that reduced neurogenesis in the hippocampus, without affecting general health of the animals. Therefore, mice (n=3 per group) received s.c. daily injections of MAM at doses of either 3, 5 or 7 mg/kg (in 0.9% NaCl) for 14 days (Fig. 1A). The body weight of the mice was monitored daily throughout the 14-day period of injections and locomotor activity was analyzed in an open-field on days 1, 7 and 14 using videotracking and computer software (Noldus, Ethovision). On days 11 and 12, 5-bromo-2'deoxyuridine (BrdU) (100 mg/kg) was administered to the mice which were sacrificed on day 15 for measurement of hippocampal neurogenesis (Fig. 1A). We found that 14 days of daily treatment with 5 mg/kg of MAM was more efficient than the 3 mg/kg dose in reducing the number of adult-generated cells in the dentate gyrus (211±12 BrdU-labeled cells in saline mice; 145±4.2 in 3 mg/kg group; 112±14 in 5 mg/kg group; 55±7.1 in 7 mg/kg group) ($F_{(3,8)}$=4.77; P<0.05; saline vs. 3 mg/kg P=0.156, saline vs. 5 mg/kg P<0.05), inducing a 47% reduction of BrdU labeled cells in treated mice compared to control mice. Moreover, treatment with either 3 or 5 mg/kg of MAM did not elicit any significant weight loss (body weight on day 14 expressed as a % of initial body weight: saline group: 100.8±0.5%; 3 mg/kg group: 99.6±2.0%; 5 mg/kg group:

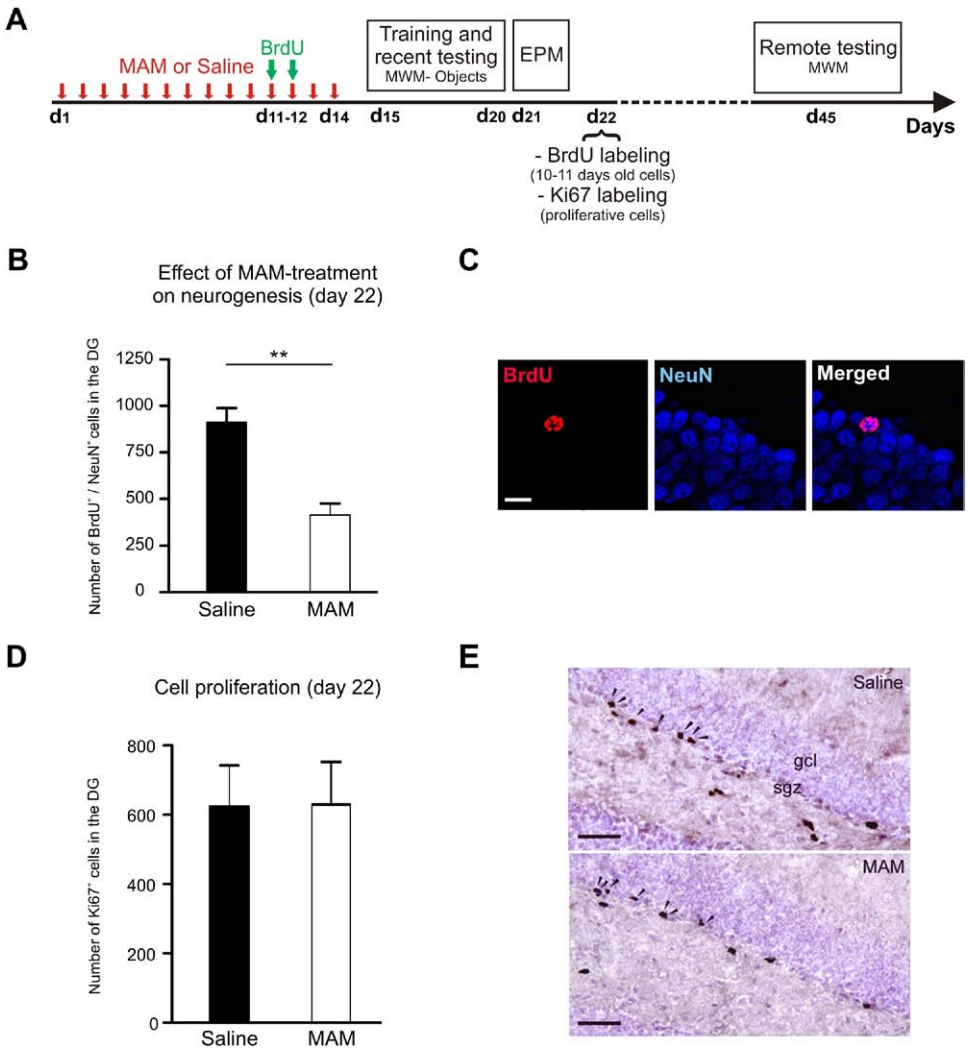

Fig. 1. MAM-treatment reduces hippocampal neurogenesis. (A) Experimental timeline. Mice were injected with methylazoxymethanol acetate (MAM) or vehicle solution (saline) for 14 d (d1–d14) and received two injections of BrdU on days 11 and 12. Mice were tested in the spatial or cued water maze (MWM), followed by testing in either the object location or object recognition tasks (Objects). After testing in the elevated plus maze (EPM), all mice were sacrificed for immunohistochemistry, excepted an independent group of mice which was tested for remote memory in either the spatial or cued water maze (remote testing MWM) 30 d after training, on day 45. (B) The total number of double BrdU/NeuN-labeled ($BrdU^+/NeuN^+$) cells was reduced by 59% in the dentate gyrus (DG) of MAM-treated mice compared to saline control mice (** P<0.01, Saline vs. MAM mice, values are presented as mean±SEM). (C) Confocal analysis was used to score the co-expression of NeuN (blue) in BrdU+ cells (red) (Bar=15 μm). (D) Similar numbers of proliferating Ki67-labeled ($Ki67^+$) cells were found in the DG of MAM-treated mice and control animals, indicating that cell proliferation is intact after completion of behavioral testing (on day 22) in MAM-treated mice (mean values±SEM). (E) Photomicrographs depicting Ki67-labeled cells (arrowheads) in the DG of saline and MAM-treated animals (sgz, subgranular zone; gcl, granular cell layer; Bar=50 μm). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

93.9±5.0%; $F_{(3,8)}=3.753$; $P=0.06$; *post-hoc* LSD saline vs. 3 mg/kg: 0.766; saline vs. 5 mg/kg: 0.129) in contrast to the highest dose of 7 mg/kg (88.7±2.0% of initial body weight, *post-hoc* LSD saline vs. 7 mg/kg: $P<0.05$). Finally, we did not observe changes in locomotor activity (total distance moved in peripheric and central zones of a square open-field) during treatment with MAM, at either dose of 3, 5 or 7 mg/kg ($F_{(3,8)}=1.167$; $P=0.380$). Therefore, the dose of 5 mg/kg of MAM was used in the subsequent study.

Behavioral testing

Behavioral testing began on day 15 and lasted for six days (Fig. 1A). Each mouse was subjected to either the spatial or cued-version of the water maze, and to either the object location or the novel object recognition test. Within each group of mice, the behavioral tests occurred in a counterbalanced order to avoid any bias. No effect due to the sequence of tests was found.

Spatial navigation in the water maze. The spatial version of the water maze was used to investigate hippocampal-dependent spatial navigation. The maze consisted of a circular pool (110 cm diameter and 30 cm high), filled to a depth of 15 cm with opaque water (23.5 °C±0.5 °C). A circular escape platform (diameter, 9 cm) was located in the centre of one quadrant (i.e. target quadrant) and remained at a fixed location during training. White curtains affixed with large extramaze visual cues surrounded the pool which was divided into four virtual quadrants. The three other quadrants (opposite, adjacent 1 and 2) contained the starting points which were used in a pseudo-randomized order that varied across blocks of training trials.

The general procedure of this test has been described in Sargolini et al. (2003). Briefly, it consisted of three different phases: familiarization, training and probe-test. The day before training, animals were submitted to a single block of familiarization, consisting of three trials with the platform visible protruding 0.5 cm above the water surface. The maximal duration for each trial was 60 s after which animals that failed to find the platform were guided to it and allowed to remain on it for 60 s before being replaced in the water from another starting point. The following day, the mice were trained to locate a hidden platform (MAM: $n=17$, Saline: $n=18$). The procedure was the same as during familiarization, except that the platform still located at the same position, was submerged 0.5 cm beneath the water surface. Each mouse was submitted to four consecutive blocks of three trials with an inter-block resting period of 15–20 min during which it was returned to its home cage. The mouse had to find the invisible platform using extra-maze cues. To test memory retention of spatial orientation, mice were subjected to a single probe test without the platform, either 1 day (recent memory, MAM: $n=9$, Saline: $n=9$) or 30 days (remote memory, MAM: $n=8$, Saline: $n=9$) after training. The mouse was placed in the centre of the pool and allowed a 60 s search for the platform.

Cued navigation in the water maze. The cued version of the water maze was used to investigate hippocampal-independent non-spatial navigation. Spatial cues around the swimming pool were removed and a ball was hung above the mobile platform, indicating its location. The training phase followed the procedure described above, except that the platform and ball location was changed after each block of three trials to avoid any preference for a particular quadrant (MAM: $n=20$, Saline: $n=20$). Either 1 day (recent memory, MAM: $n=10$, Saline: $n=10$) or 30 days (remote memory, MAM: $n=10$, Saline: $n=10$) after training, a single probe test was conducted without the platform and with the ball hanging in the quadrant opposite to the one of the last training trial. The mouse was placed in the centre of the pool and allowed a 60 s search for the platform.

Water-maze data analysis. Video tracking software (Ethovision, Noldus) was used for automatic recording of a variety of parameters. Trials were collapsed into blocks of three trials for data analysis. During training, distances swum to reach the platform were analyzed and the average swimming speeds were calculated. During the probe test, the number of annulus crossings, that is number of times the mouse crossed a virtual circle (12 cm in diameter) located around each putative platform position in the four quadrants was calculated. Number of annulus crossings was reported to reflect spatial memory for the platform location more accurately than the percentage of time spent in the target quadrant during the probe test, which overestimates animals' spatial ability (Blokland et al., 2004).

Object location task. The object location test addresses the ability of rodents to discriminate between a novel and a familiar spatial location. Rats with hippocampal lesions are impaired in this one-trial object place location task (Ennaceur et al., 1997; Mumby et al., 2002). This task is presented in a square open-field (50×50×30 cm^3) surrounded by a white curtain. A conspicuous striped pattern (36×25 cm^2; black and white 1.5 cm wide vertical stripes) was placed on one wall of the open-field. One day before acquisition, each mouse was left for 10 min in the empty square open-field surrounded by a white curtain, as a familiarization (MAM: $n=7$, Saline: $n=9$). The next day, the exploration phase took place and two identical objects were placed in the middle of the open-field. The mice were allowed to explore for 10 min during which the time spent exploring the two objects was recorded. In the test phase held 1 day later, identical copies of the sample objects were exposed and one of the two objects was moved to a novel location. The position (left or right) of the displaced object was chosen pseudorandomly to reduce bias towards a particular position. Mice were allowed to explore the objects during 10 min. The open field was cleaned thoroughly between the trials of each mouse to ensure the absence of olfactory cues.

Object recognition task. This task is based on the spontaneous preference of rodents for novelty and their ability to remember previously encountered objects (Ennaceur and Delacour, 1988; Dodart et al., 1997). This task assesses the ability of mice to discriminate between a novel and a familiar object and does not require the full integrity of the hippocampus (Ennaceur et al., 1997; Gaskin et al., 2003; Winters et al., 2004). The same open-field with the same environment as the object location task was used except that the striped pattern was removed. The familiarization session was identical to the object location task described above (MAM: $n=9$, Saline: $n=9$). The next day, the exploration phase took place and two identical objects were placed in the middle of the open-field. The time the animal spent exploring each object was recorded. We ensured that every mouse spent the same time exploring the objects and avoided any bias due to differences in individual levels of exploration by removing the animal once it had explored the objects for a total of 30 s. Animals that did not reach this criteria within 10 min were excluded. Recognition memory was tested 1 day after the exploration phase. Mice were reintroduced into the arena and exposed to two objects, a familiar object and a novel object, whose positions were identical to session 1. The familiar object was a triplicate copy of the sample used in session 1, in order to avoid olfactory trails. The mouse was allowed to explore for 10 min during which, the time spent exploring each object was recorded. The nature and position (left or right) of the new object was randomized to reduce bias towards a preference for a particular object or location. The open field was cleaned thoroughly between each mouse to ensure the absence of olfactory cues.

Objects data analysis. Measurement of the time spent exploring the novel or displaced object was expressed as a percentage of the exploration time of the novel or displaced object related to the total exploration time for both objects during the test phase (preference index).

Locomotor activity in the open field. Locomotor activity was analysed during the 10 min familiarization phase of the object

recognition or object location tasks (MAM: $n=11$, Saline: $n=13$). The open-field was divided into centre (central square, 30×30 cm^2) and periphery (a 10 cm width band starting at the wall) sectors. The distance moved in each sector was measured and the percentage of time spent in the centre was calculated.

Elevated plus maze. The day after mice had completed the learning and memory tasks (day 21), their potential differential anxiety-related behavior was evaluated for each group (MAM: $n=7$, Saline: $n=6$) using the elevated plus maze task (Fig. 1A). This test is classically used to measure anxiety-related behavior in mice (Crawley, 2008). The maze consisted of a plus-shaped track, with two closed ($30\times5\times15$ cm^3) and two open (30×5 cm^2) arms that extended from a common central platform (5×5 cm^2). The apparatus was located in a room with dim light and bare walls and elevated 50 cm above the floor. Each trial began with the mouse placed in the centre facing an open arm and lasted 5 min. For each mouse, the ratio of time spent in the open arms divided by the total time spent in both open and closed arms was calculated and expressed as a percentage. We also calculated the percentage of entries onto the open arms (number of entries in open arms divided by the total number of entries in the four arms) as an index of anxiety. Entries into an arm were only counted if mice entered it with all four paws. The maze was cleaned between each mouse to remove olfactory cues.

Statistical analysis of behavioral data

SYSTAT 11.0 statistical software package was used for data analysis. The results were expressed as mean±SEM and analyzed using one or two-way ANOVAs, or a repeated measure ANOVA when appropriate. *Post-hoc* multiple comparisons were carried out when allowed, using Tukey's Honestly Significant Distance (HSD) test.

Tissue preparation and immunostainings

The day after the end of behavioral testing (day 22, i.e. 10 days after the last BrdU injection), animals were deeply anaesthetized and perfused transcardially with 0.1 M phosphate buffer (0.1 M PB, pH 7.4) (PB) followed by 4% paraformaldehyde in 0.1 M PB, at 4 °C. The brains were left in the fixative overnight, transferred into 30% sucrose and cut into coronal sections (30 μm thick) on a cryostat. For each experiment, sections incubated without the primary antibody remained virtually free of immunostaining and served as controls. All counts were conducted by an experimenter blind to the experimental conditions.

BrdU immunohistochemistry

Series of one in six free-floating sections were rinsed extensively in PBST before quenching endogenous peroxidases with 3% H_2O_2 and 10% methanol in PBS. Sections were then treated in order to denature the DNA (2N 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 rat monoclonal anti-BrdU (1:400, OBT0030, Harlan Seralab) in PBST containing 5% normal goat serum. The next day, sections were incubated in biotinylated goat anti-rat antisera (1:400, Vector laboratories) for 90 min, rinsed and incubated in avidin-biotin-peroxidase complex (1:400, Vector ABC Elite Kit) for 90 min. Following peroxidase detection in a solution of 3,3′-diaminobenzidine containing 0.06% nickel ammonium sulfate (DAB Kit, Vector Laboratories), sections were mounted onto slides, counter-stained and cover-slipped.

Neuronal phenotypic determination of BrdU-labeled cells. To determine the proportion of newborn cells that adopted a mature neuronal phenotype, sections were double-labeled for BrdU and Neuron-specific Nuclear protein (NeuN), a marker of postmitotic neurons. Series of 1 in 12 sections (360 μm spacing) spanning the entire hippocampus were pretreated and denatured as described above before being blocked in 5% Normal Goat Serum (NGS) in PBST for 1 hour at room temperature. Sections were then incubated overnight at room temperature in a mix of anti-BrdU antibody (1:400) and monoclonal mouse anti-NeuN (1:2000, MAB377; Chemicon, Temecula, CA, USA) in PBST containing 5% NGS. The next day, sections were rinsed several times in PBST before being incubated for 90 min at room temperature in a mixture of secondary reagents: biotinylated goat anti-rat antiserum (1:400; Vector Laboratories) and Alexa 647 conjugated goat anti-mouse IgG (1:400; Invitrogen) in PBST. Sections were rinsed again and finally incubated 120 min in streptavidin–tetramethylrhodamine isothiocyanate (TRITC; 1:1000 in PBST; Beckman Coulter, Fullerton, CA, USA). Sections were mounted onto subbed slides, coverslipped using Mowiol, and stored at 4 °C.

Ki67 immunohistochemistry. Evaluation of proliferative activity was performed using an antibody directed against Ki67, a nuclear protein expressed in all phases of the cell cycle except the resting phase. Series of one in six sections were rinsed extensively in PBST before quenching endogenous peroxidases with 3% H_2O_2 and 10% methanol in PBS. Sections were then incubated overnight in rabbit anti-human Ki67 antibody (1:500, NCL-Ki67p, Novocastra Laboratories, Vision BioSystems, Newcastle Upon Tyne, UK). The next day, sections were incubated in biotinylated goat anti-rabbit antisera (1:400) for 90 min, rinsed and incubated in avidin-biotin-peroxidase complex (1:400, Vector ABC Elite Kit) for 90 min. Final reactions were conducted as described for BrdU staining and sections were mounted as above.

Quantification and image analysis

Stereological quantification of BrdU-labeled or Ki67-labeled cells (BrdU+ and Ki67+ respectively) was conducted bilaterally on every six section (180 μm apart) through the rostro-caudal extent of the hippocampus. The density of labeled cells was calculated by dividing the number of BrdU+ or Ki67+ cells by the gcl/sgz zone sectional volume measured with the Mercator morphometric system (Explora Nova). Finally, total numbers of BrdU+ or Ki67+ cells were obtained by multiplying BrdU+ cell density by the reference volume as previously described (Trouche et al., 2009).

The proportion of mature neurons among the BrdU-labeled cell population was measured in series of sections double-labeled for BrdU and NeuN. Twenty-five BrdU+ cells per animal were randomly analyzed for coexpression with NeuN using a confocal laser-scanning microscope (TCS SP2; Leica, detailed informations in Trouche et al., 2009). Absolute numbers of double-labeled BrdU/NeuN cells for control and MAM groups of mice were determined by multiplying the neuronal fraction by the total number of BrdU+ cells for each animal. Photographs were processed using Adobe Photoshop software 7.0 (Adobe System).

Statistical analysis of the cellular data

SYSTAT 11.0 statistical software package was used for data analysis. The results were expressed as mean±SEM and analyzed using one way ANOVA. *Post-hoc* multiple comparisons were carried out when allowed, using Fisher LSD's test.

RESULTS

Hippocampal neurogenesis is reduced by MAM treatment during behavioral tests

Mice were sacrificed after completion of the behavioral tests (day 22), when BrdU-labeled (BrdU+) cells were 10 to 11 days old (Fig. 1A). Quantification of double-labeled BrdU+/NeuN+ cells revealed that the dentate gyrus of MAM-treated mice contained a significantly lower number

of new neurons (368.1±86.4) compared to control mice (896.6±107.6) ($F_{(1,15)}$=14.192; P<0.01; Fig. 1B, C). Thus, the subchronic treatment with MAM reduced by 59% the population of hippocampal neurons younger than 14 days of age. In order to evaluate the status of hippocampal cell proliferation after completion of behavioral testing, we examined the expression of Ki67, an endogenous marker of proliferating cells (Kee et al., 2002). We found similar a number of proliferating Ki67-labeled (Ki67+) cells in the dentate gyrus of MAM-treated and saline control mice (629.6±121.6 and 626.0±116.6 Ki67+ cells respectively; $F_{(1,10)}$=0.001; P=0.983; Fig. 1D, E) 8 days after the end of MAM-treatment.

Reduced hippocampal neurogenesis does not affect performances during spatial training in the Morris water maze

To determine the consequences of hippocampal neurogenesis reduction on spatial memory, we used the hippocampal-dependent version of the Morris water maze task. Across training trials, all mice showed a decrease in the length of their swim path to reach the escape platform (one-way ANOVA with repeated measures: $F_{(3,99)}$= 13.835; P<0.001) (Fig. 2A). Our data indicate that MAM treatment did not affect this decrease ($F_{(1,33)}$=1.263; P=0.269). Moreover, shortening of the distance swam to reach the hidden platform occurred at a similar rate in control and MAM-treated groups of mice ($F_{(3,99)}$=1.319; P=0.272). Finally, MAM treatment did not produce any general effect on swim speed during acquisition (Saline: 17.70±0.64 cm/s; MAM: 18.55±0.75 cm/s; $F_{(1,33)}$=2.934; P=0.096) and no interaction was found between treatment and training session ($F_{(3,99)}$=0.502; P=0.682).

Reduced hippocampal neurogenesis impairs recent and remote spatial memory in the Morris water maze

Mice were randomly tested either 1 day (recent memory) or 30 days (remote memory) after acquisition in the water maze (Fig. 2B, C). During the 1 day probe test, no overall difference between control and MAM mice was found regarding the total number of annulus crossings (ANOVA, $F_{(1,64)}$=0.994, P=0.323) (Fig. 2B). However, a significant annulus effect ($F_{(3,64)}$=25.078, P<0.001) and an interaction between treatment and annulus crossings ($F_{(3,64)}$= 3.873, P<0.05) were observed. Interestingly, a *post-hoc* comparison indicated that saline animals crossed significantly more often the target annulus where the platform had been located during acquisition, than the other three annuli (P<0.001; Fig. 2B). Although MAM-treated mice showed a preference for the target annulus compared to

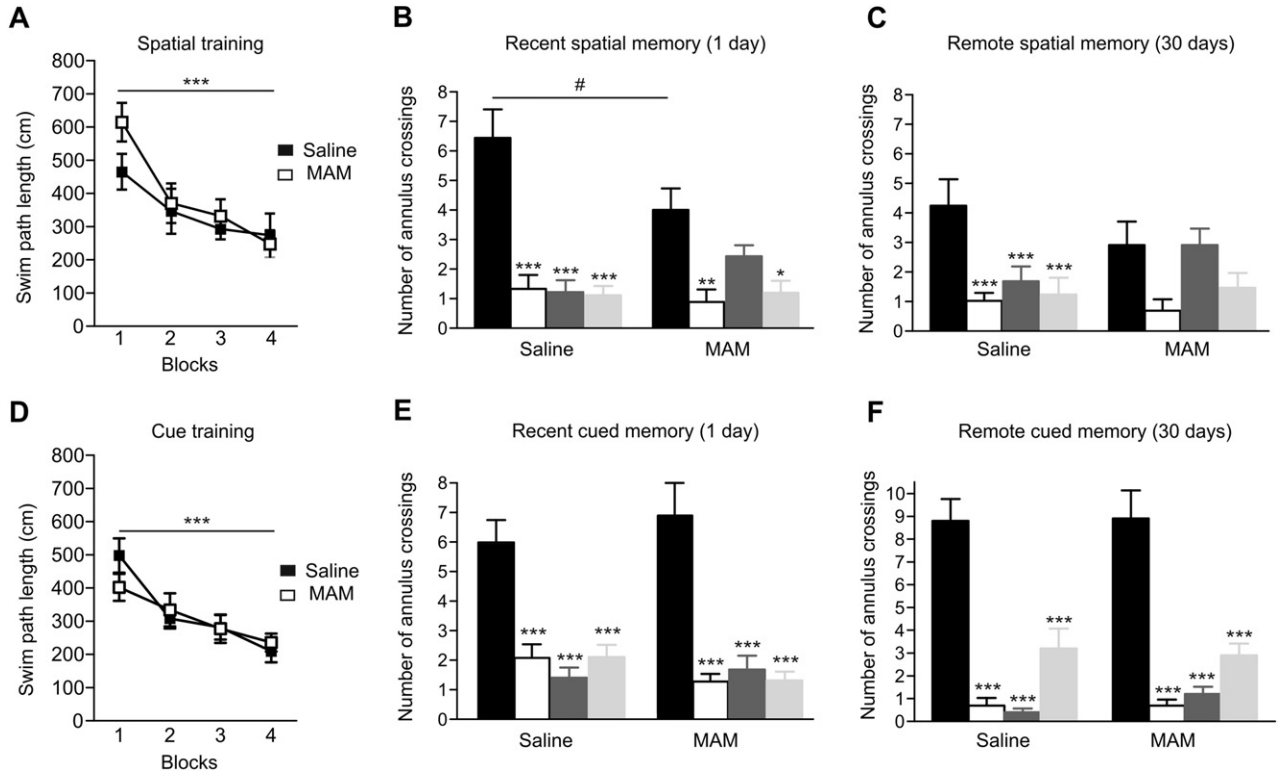

Fig. 2. Neurogenesis reduction impairs spatial memory but spares non-spatial memory in the Morris Water maze. (A) Saline and MAM-treated groups of mice showed similar performances during training to locate the hidden platform in the spatial water maze (*** P<0.001). Deficits in recent (B) and remote (C) spatial memory were found in the MAM-treated group. (D) Neurogenesis reduction by MAM had no effect on performances during training in the cued water maze, nor on recent (E) and remote (F) memory in this task (*** P<0.001). In (A) and (D), each dot represents the mean distance (±SEM) swum to reach the hidden platform across one block of three trials. Performances during recent (B, E) and remote probe tests (C, F) are expressed as mean numbers of annulus crossings±SEM (* P<0.05, ** P<0.01, *** P<0.001 target annulus vs. others; # P<0.05, target annulus in saline vs. MAM). Quadrants: Target, Opposite, Adjacent 1, Adjacent 2.

the opposite ($P<0.01$) and adjacent 2 ($P<0.05$) annuli, the number of annuli crossings in the target quadrant was significantly reduced in MAM-treated animals compared to controls ($P<0.05$) (Fig. 2B).

To evaluate whether reduction of hippocampal neurogenesis prior to spatial learning produces long-term consequences on spatial memory, two independent groups of mice were tested 30 days after task acquisition in the water maze (Fig. 2C). An ANOVA revealed no general effect of MAM-treatment ($F_{(1,60)}=0.330$; $P=0.568$), but a significant annulus effect ($F_{(3,60)}=8.663$; $P<0.001$) and more importantly, an interaction between treatment and annulus crossings ($F_{(3,60)}=3.541$; $P<0.05$). Thus, during remote memory testing, control mice crossed the target annulus more often than the three other annuli ($P<0.001$; Fig. 2C) indicating that they remembered precisely where the hidden platform was located during training. In contrast, MAM-treated animals showed no preference for the target annulus compared to the other three annuli ($P>0.5$; Fig. 2C), indicating long-term memory impairment in these mice.

Performances in the cued Morris water maze task are insensitive to hippocampal neurogenesis reduction

To establish whether behavioral effects of reduced hippocampal neurogenesis are specific for spatial memory, we used the non-spatial version of the Morris water maze. Mice of both groups rapidly learned to locate the visible platform as revealed by the highly significant overall decline in swim-path length during task acquisition ($F_{(3,114)}=13.074$; $P<0.001$) (Fig. 2D). Neurogenesis reduction by MAM did not affect performance level ($F_{(1,38)}=0.007$; $P=0.936$) nor acquisition rate ($F_{(3,114)}=1.437$; $P=0.236$) during cued training (Fig. 2D). Memory was assessed either 1 day (recent memory) or 30 days (remote memory) after acquisition in the cued water maze. During recent and remote probe tests, saline and MAM-treated mice demonstrated a strong preference for the target annulus where the platform had been located during task acquisition (recent testing $F_{(3,72)}=34.677$; $P<0.001$; remote testing $F_{(3,72)}=61.350$; $P<0.001$) (Fig. 2E, F). At both delays, no effect of MAM-treatment (1 day: $F_{(1,72)}=0.060$; $P=0.807$; 30 days: $F_{(1,72)}=0.094$; $P=0.760$) and no interaction between treatment and annulus exploration (1 day: $F_{(3,72)}=1.073$; $P=0.366$; 30 days: $F_{(3,72)}=0.227$; $P=0.877$) were found.

In summary, reduction of hippocampal neurogenesis alters both recent and remote spatial memory but has no effect on non-spatial memory. To investigate whether these findings could be generalized to other memory tasks, we used the object location and the object recognition tests. Unlike the water maze, these tasks are non-aversive, non-navigational, non-motivational and their acquisition takes place during a single trial.

Reduced hippocampal neurogenesis impairs spatial memory in the object location task

The object location task addresses the ability of rodents to discriminate between a novel and a familiar spatial location. During the exploration phase, MAM-treated and control mice spent the same amount of time exploring each of the objects (34.14±5.75 s and 32.60±1.57 s respectively, $F_{(1,14)}=0.082$ $P=0.778$). Importantly, the average time spent exploring each object was the same independently of the pairs of objects, indicating that these objects elicited similar interest in the animals (data not shown). One day after the exploration phase, one of the objects was moved to a novel location in the arena and spatial memory was tested. Saline control mice spent significantly more time exploring the object that had been displaced than the object that had remained in the familiar location. As expected, the exploratory preference index for control mice was clearly different from chance level (50%) (59.169±2.54%; $P<0.01$, Fig. 3A). In contrast, MAM-treated mice spent an equal amount of time exploring each object, indicating that they were unable to discriminate the novel from the familiar location. In line with this, the exploratory preference index for MAM-treated mice was not different from chance (51.50±3.92%; $P=0.714$, Fig. 3A).

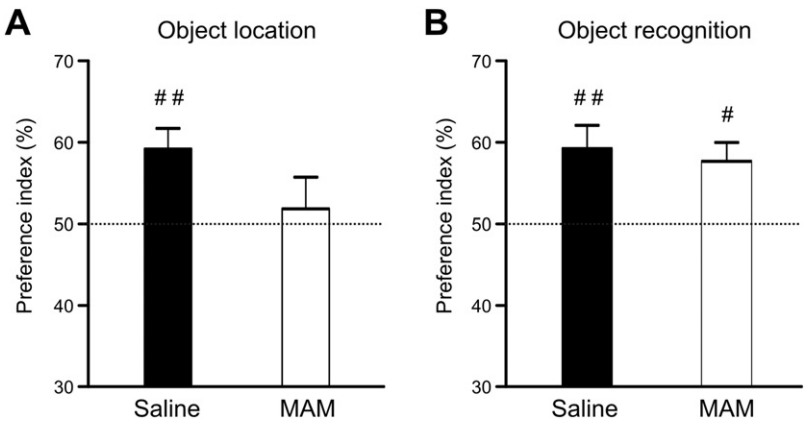

Fig. 3. Neurogenesis reduction impairs hippocampal-dependent object location memory but spares hippocampal-independent object recognition memory. Performances of saline and MAM-treated mice in the object location task (A) and in the object recognition task (B) are expressed as the group mean (±SEM) preference index (% of time exploring the displaced (A) or novel (B) object, related to the total exploration time for both objects). Horizontal dotted lines represent equal exploration of the two objects ($^{\#}P<0.05$; $^{\#\#}P<0.01$; index vs. chance level (50%)).

Non-spatial recognition memory is insensitive to hippocampal neurogenesis reduction

Finally, we used the object recognition task to examine whether new hippocampal neurons participate in the formation of non-spatial memory. During the exploration phase, all animals spent the same time exploring the objects (inclusion criteria of 30 s total exploration time, see Methods). Recognition memory was tested 1 day after exposure to the objects, when one of the familiar objects was replaced by a new one. During the retention test, all animals spent significantly more time exploring the novel object than the familiar one, so that the preference index was significantly different from chance level (50%) (saline: 58. 58 ±1.34%; $P<0.01$, MAM: 57.51±2.30%; $P=0.013$; Fig. 3B). These results indicate that both groups of mice remembered the object they had encountered one day earlier, despite hippocampal neurogenesis reduction induced prior to acquisition.

Both object location and object recognition tasks are based on the animal's spontaneous preference for novelty and require the animals to explore the objects. Thus, the fact that MAM-treated mice were specifically impaired in the object location but not in the object recognition task, ruled out a putative non-specific effect of MAM-treatment on animal's exploratory behavior.

MAM-treatment has no effect on anxiety-like behaviors

Since we cannot exclude that increased anxiety may alter memory performances in our spatial memory tasks, we investigated the effect of MAM-treatment on anxiety-related behavior in mice. Using the elevated plus-maze, we measured behavioral propensity for avoidance responses during a risk-associated situation and exploratory behavior. We found that treatment with MAM did not affect the percentage of time spent in the open arms (saline 37.1±4.1%, MAM 31.9±2.5%; $F_{(1,11)}=1.234$, $P=0.290$) nor the percentage of entries in the open arms (number of entries in open arms/total number of entries in the four arms, an index of anxiety) (saline 45.2±3.0%, MAM 44.35±2.7%; $F_{(1,11)}=0.042$, $P=0.840$). Additionally, the percentage of time spent in the centre of the arena during the familiarization phase of the object recognition or object location tasks was evaluated as an index of anxiety. We found that MAM-treated mice spent the same amount of time in the centre of the arena as control mice (saline: 25.6±1.3%, MAM: 23.2±2.6%; $F_{(1,22)}=0.729$, $P=0.403$). However, and in contrast to our preliminary data obtained from a small number of animals (see Methods, dose 5 mg/kg, $n=3$), we found that MAM-treated mice exhibited a reduced locomotor activity (total distance moved in an open-field: saline: 5315±225 cm, MAM: 4024±333 cm; $F_{(1,22)}=10.82$; $P<0.01$).

Altogether, these data indicate that neurogenesis reduction by MAM did not induce specific anxiety-like behavior. Such findings rule out the possibility that the behavioral impairments reported above may result from anxiogenic effects or from differential emotional reactivity due to the pharmacological treatment.

DISCUSSION

In the present study we report that a reduced number of young hippocampal neurons leads to memory impairment depending both on the type of task (spatial vs. non-spatial) and on the task's dependence on the hippocampus (dependent vs. independent). Our findings demonstrate that spatial hippocampal-dependent types of memory are vulnerable to a partial reduction of adult neurogenesis.

To reduce neurogenesis in the hippocampus, we used the cytostatic agent MAM which reversibly stops progenitor cells from dividing (Shors et al., 2001; Bruel-Jungerman et al., 2005). High doses of MAM used to achieve nearly complete reduction of adult neurogenesis have been shown to induce cachexia (Dupret et al., 2005). We therefore determined and used a lower dose of MAM which only induced a partial reduction of the production of new cells, but did not alter the general health of the animals. MAM was administered to the mice during the two-week period preceding behavioral training and resulted in a partial but significant (59%) reduction of the number of new neurons in the dentate gyrus. Then, the putative role of young hippocampal neurons was assessed using spatial and non-spatial tasks in the water maze where we addressed both recent and remote memory. Despite a residual fraction (41%) of newborn cells in the dentate gyrus, we found that MAM treated animals exhibited memory impairments, thus highlighting their crucial contribution to these processes. Our findings are in line with previous studies that have used either a pharmacological compound, X-ray irradiation or genetic approaches to reduce adult neurogenesis and have reported intact performances in spatial or non-spatial water maze tasks (Shors et al., 2002; Madsen et al., 2003; Raber et al., 2004; Rola et al., 2004; Snyder et al., 2005; Meshi et al., 2006; Clark et al., 2008; Wojtowicz et al., 2008; Jessberger et al., 2009). However, others studies have found that a severe depletion of newborn hippocampal neurons using pharmacological (Garthe et al., 2009) or genetic (Dupret et al., 2008; Zhang et al., 2008) methods in mice, led to subtle and specific spatial learning deficits in the spatial water maze task. Although we found that all mice reached the same level of performances at the end of training, we cannot rule out that subtle differences in spatial learning may exist between groups of mice. A more thorough analysis of the animal's search strategies may be useful to examine this possibility (Garthe et al., 2009). Another explanation for this discrepancy may come from the fact that our study differed from those of Dupret et al. (2008) and Garthe et al. (2009) regarding the familiarization (the platform remained at the same position during familiarization and training in our study) and training procedure (distributed vs. pseudo-massed in our study). Most importantly, these reports differ from ours by the age of adult-born neurons that were depleted at the time of behavioural testing. In the present work, we examined the contribution of 1 to 2 week-old

cells, which corresponds to a time-window when these cells are still immature. In contrast, previous reports focussed on a population of new cells that were at least 4 weeks-old at the time of training in the water maze (Dupret et al., 2008; Zhang et al., 2008; Garthe et al., 2009), corresponding to a time period that is required for the maturation of new neurons. Thus, our results suggest that new granule neurons, during the first and second weeks after their birth, may not be critically involved in spatial task acquisition. In contrast, we found that the lack of young neurons at the time of training leads to recent and remote spatial memory impairments. Although it was reported that a severe abolition of neurogenesis by irradiation induced memory impairment one month after training (Snyder et al., 2005), other reports indicated that spatial memory was not affected by such neurogenesis reduction when tested at shorter delays ($<$1 day) after training in the water-maze (Shors et al., 2002; Raber et al., 2004; Wojtowicz et al., 2008; Meshi et al., 2006). Using inducible TLX-deficient mice, Zhang et al. (2008) reported that learning was delayed in these mice which exhibit impaired short-term spatial memory but intact long-term memory. In the present work, using the number of annuli crossings as memory performance criteria, we found that a moderate reduction of neurogenesis impaired the precision of the search in the pool during both recent and remote probe tests. Our results suggest that in the lack of new neurons, the recent memory that is formed is less detailed and becomes more easily degraded over time, leading to forgetting one month later. Such critical involvement of the young neurons present during task acquisition, in the consolidation and/or the recall of recent spatial memory, is supported by a neurocomputational model of hippocampal neurogenesis demonstrating that new neurons enable an important increase in the capacity of the hippocampus to retrieve recent memories (Weisz and Argibay, 2009). Altogether our data suggest that new neurons, as they reach the first and second weeks after their birth, may not critically contribute to task acquisition, but become later involved in memory function. Thus, in normal conditions of neurogenesis, newborn neurons may be offline at the time of initial learning, but may be receptive to surrounding neuronal activity elicited in the dentate gyrus, in response to performing tasks that solicit hippocampal networks. The present results corroborate our demonstration that the recruitment of young neurons into hippocampal networks supporting spatial memory is determined as early as during task acquisition, and may be mediated by a tagging process which remains to be identified (Trouche et al., 2009). Supporting this idea, it was recently found that the dendritic maturation of 1 to 2 week-old neurons is influenced by spatial learning in the water maze (Tronel et al., 2010). These data indicate that despite their relative immaturity—at this age adult-generated neurons lack glutamatergic inputs (Esposito et al., 2005; Zhao et al., 2006)—young neurons are receptive to ongoing hippocampal neuronal activity and might contribute to post-learning processes such as consolidation and/or recall of hippocampal-dependent memory, via their activity-dependent structural plasticity. In line with this idea, Farioli-Vecchioli et al. (2008) have used a mouse model in which the differentiation of adult-generated granule neurons can be accelerated and have provided elegant evidence that the contribution of new neurons to memory processes is critically dependent on their maturational state.

The Morris water maze is based on the use of a negative reinforcement (i.e. the immersion in the water) which may generate stress (Sandi et al., 1997) and in turn, might downregulate hippocampal neurogenesis. In order to dispel putative influence of stress on our findings, we further addressed the role of young neurons using spatial and non-spatial memory paradigms which do not require reinforcers, such as the novel object location and novel object recognition tasks respectively. In these tasks, the stress elicited is noticeably lower than in the Morris water maze procedure (Sandi et al., 1997; Okuda et al., 2004). We found that a moderate reduction of hippocampal neurogenesis was sufficient to impair memory in the object location task, which relies on the hippocampus (Mumby et al., 2002). Indeed, in contrast to control mice, animals with a reduced number of young neurons did not respond to the change in spatial configuration of the objects when they are tested one day after the exploration phase. Raber et al. (2004) showed that immediately after exploration, irradiated mice were able to detect a change in the location of a familiar object, suggesting that the impairment found in our MAM-treated mice is merely due to a deficit in memory and/or its expression, rather than to impaired learning. Theoretical models, along with experimental data, have provided insights of how the granule cell population in the dentate gyrus may contribute to pattern separation, a process whereby the hippocampus forms distinct representations for events with similar features (Rolls, 1996; Leutgeb et al., 2007; McHugh et al., 2007). Our findings that newborn cells are required for the memorization and/or the recall of spatial relationships between objects are in line with a recent work by Clelland et al. (2009) reporting that newborn neurons may be necessary for normal pattern separation function in the DG of adult mice.

We then examined whether neurogenesis reduction had an impact on the novel object recognition task. Performances in this non-spatial task, in contrast to the object location test, are not affected by hippocampal lesions (Ennaceur et al., 1997; Gaskin et al., 2003; Winters et al., 2004) unless the test is held in a complex spatial environment (Winters et al., 2004). To avoid this bias, we used the original procedure described by Ennaceur and Delacour (1988), which does not require an intact hippocampus, as we previously demonstrated (Stupien et al., 2003). Despite the reduced number of young neurons in the dentate gyrus, we found that mice spent more time exploring the novel object than the familiar one, indicating intact recognition memory one day after exploration. These findings extend to a longer delay those of Raber et al. (2004) and Madsen et al. (2003) on short-term memory. Using a hippocampus-dependent version of object recognition task, Jessberger and collaborators (2009) described a dose-dependent effect of neurogenesis depletion on memory

retention. They found that a slight (15%) depletion of hippocampal neurogenesis had no effect on recognition memory when tested 3 h and 1 month after training, whereas a higher degree of neurogenesis knockdown (85%) impaired recognition memory retrieval (Jessberger et al., 2009). Here we found that the presence of a restricted number of immature neurons (41%) at the time of task acquisition produced an impairment of the mice ability to form memory of spatial configurations, but had no effect on recognition memory.

CONCLUSION

Altogether, our findings support the idea that newborn hippocampal neurons aged between 1 and 14 days are required for hippocampal-dependent forms of memory. These data are in agreement with our recent report that new neurons contribute to remote spatial memory retrieval (Trouche et al., 2009) and further point out that they are critically involved in recent spatial memory. Although adult-generated granular neurons represent only a small population of cells within the adult dentate gyrus, increasing evidence indicates that they seem ideally suited for processing specific experience-related inputs during learning and for modulating post-learning processes such as memory consolidation. The present results extend the range of tasks that can be said to be vulnerable to the effects of neurogenesis suppression. They provide additional evidence on the role played by adult-generated neurons in hippocampus-dependent memory processes, as a function of their age and learning context.

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