

Hippocampus-Dependent Learning Is Associated With Adult Neurogenesis in MRL/MpJ Mice

Sandrine Thuret,^{1,2} Nicolas Toni,¹ Stefan Aigner,¹ Gene W. Yeo,¹ and Fred H. Gage^{1*}

ABSTRACT: The hippocampus is involved in declarative memory and produces new neurons throughout adulthood. Numerous experiments have been aimed at testing the possibility that adult neurogenesis is required for learning and memory. However, progress has been encumbered by the fact that abating adult neurogenesis usually affects other biological processes, confounding the interpretation of such experiments. In an effort to circumvent this problem, we used a reverse approach to test the role of neurogenesis in hippocampus-dependent learning, exploiting the low levels of adult neurogenesis in the MRL/MpJ strain of mice compared with other mouse strains. We observed that adult MRL/MpJ mice produce 75% fewer new neurons in the dentate gyrus than age-matched C57BL/6 mice. Learning-induced synaptic remodeling, spatial learning, and visual recognition learning were reduced in MRL/MpJ mice compared with C57BL/6 mice. When MRL/MpJ mice were allowed unlimited access to running wheels, neurogenesis along with spatial learning and visual recognition learning were increased to levels comparable to those in running C57BL/6 mice. Together, these results suggest that adult neurogenesis is correlated with spatial learning and visual recognition learning, possibly by modulating morphological plasticity in the dentate gyrus. © 2009 Wiley-Liss, Inc.

KEY WORDS: neural stem cells; synaptic plasticity; dendritic spines; voluntary exercise; ultrastructure

INTRODUCTION

Adult neurogenesis results in the addition of new neurons in two areas of the adult mammalian adult brain, the subgranular zone of the hippocampus and the subventricular zone (Altman and Das, 1965; Kaplan and Bell, 1984; Eriksson et al., 1998; Gould et al., 1999). Although much effort has been devoted to understanding the function of adult neurogenesis in the hippocampus, the rarity of appropriate mouse models with decreased hippocampal neurogenesis has hindered conclusive

experiments. Indeed, knockout and transgenic mice showing a decrease in neurogenesis are usually unhealthy or die prematurely, because the targeted genes have additional functions in vital organs (Ferri et al., 2004; Shi et al., 2004; Filipkowski et al., 2005). Treatments that reduce neurogenesis, such as gamma irradiation of the head (Snyder et al., 2005) or injection of the anti-mitotic agent methylazoxymethanol (Shors et al., 2001; Bruel-Jungerman et al., 2005), are accompanied by inflammatory reactions and immunosuppression that are difficult to control and alter physiological and behavioral responses.

In this study, we compared the MRL/MpJ strain of mice with C57BL/6 mice. The MRL/MpJ mouse is a healthy, inbred mouse derived from the interbreeding of four lines: LG/J, AKR/J, C3H/HeJ, and C57BL/6 (Li et al., 2001). MRL/MpJ mice have a unique capacity for wound healing and tissue regeneration, as shown by the unusually rapid closure of ear hole punches (Clark et al., 1998) and the efficient regeneration of injured heart tissue, which contrasts with the scar formation seen in C57BL/6 mice (Lefterovich et al., 2001). Although the molecular bases for these phenomena remain to be investigated, genetic linkage analysis has revealed that wound healing is a complex, multigenic, and sexually dimorphic trait (McBrearty et al., 1998; Masinde et al., 2001, 2005; Blankenhorn et al., 2003; Heber-Katz et al., 2004).

These observations prompted us to investigate whether the exceptional regenerative properties of these mice might also be reflected in elevated baseline and inducible levels of adult neurogenesis. Using immunohistochemistry to assess adult neurogenesis and serial section transmission electron microscopy to analyze synaptic morphology, we were surprised to find that MRL/MpJ mice naturally show a 75% reduction in neurogenesis and an absence of learning-induced synaptic remodeling when compared with C57BL/6 mice. To analyze the learning abilities of the MRL/MpJ mice, we used two conventional types of learning and memory tests: the Morris water maze for spatial learning (Morris, 1984) and the visual-paired comparison (VPC) test for object recognition memory (Ennaceur and Delacour, 1988). Both tests require normal hippocampal function. Our results suggest that hippocampus-dependent learning depends on the

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level of adult neurogenesis, possibly through a mechanism involving synaptic remodeling.

MATERIALS AND METHODS

Animals

Ten-week-old C57BL/6 and MRL/MpJ female mice were obtained from The Jackson Laboratory. Animals were housed according to standard National Institutes of Health (NIH) regulations in regular laboratory cages, five mice per cage. Running mice were housed individually in cages containing a running wheel. Eight mice per strain were used for each of the cell proliferation and survival studies. For each behavioral study, 10 mice per strain were used; for serial section electron microscopy, nine C57BL/6 (four learners and five nonlearners) and six MRL/MpJ mice (three learners and three nonlearners) were used. All animal experimentation reported in the article was conducted in accordance with NIH guidelines and was approved by the Salk Institutional Animal Care and Use Committee.

BrdU Injections

BrdU (5-bromo-2-deoxyuridine; Sigma) was dissolved at 10 mg/ml in 0.9% NaCl and sterile-filtered at 0.22 μ m. Animals received i.p. injections of 50 μ g/g body weight, one per day for 6 days.

Microarray Analysis and Quantitative PCR Validation

Ten-week-old mice ($n = 4$ for MRL/MpJ; $n = 6$ for C57BL/6) were anesthetized [ketamine (100 mg/kg) and xylazine (10 mg/kg)]. Hippocampi were dissected in the cold and immediately disrupted in Trizol RNA extraction reagent (1 ml per hippocampus pair; Invitrogen) by trituration with a fine gauge needle. RNA was prepared as per manufacturer's instructions, dissolved in water, and frozen at -80°C . The quality of RNA preparations was verified by agarose gel electrophoresis and staining with ethidium bromide. RNA concentration was determined spectrophotometrically. The synthesis of cDNA targets and hybridization to Affymetrix (Santa Clara, CA) Mouse 430 2.0 GeneChip arrays were performed according to the manufacturer's instructions. Affymetrix CEL files obtained from scanning of the GeneChip arrays were normalized using the apt-probeset-summarize function (PLIER-MM-SKETCH) in the Affymetrix Power Tools. The gene-level normalized expression data were categorized into two sets, namely MRL and BL6. We computed t statistics for each probe set comparing MRL vs. BL6. For example, we defined the t statistic comparing MRL vs. BL6 as $t_{\text{MRL, BL6}} = (\mu_{\text{MRL}} - \mu_{\text{BL6}}) / \sqrt{((n_{\text{MRL}} - 1)\sigma_{\text{MRL}}^2 + (n_{\text{BL6}} - 1)\sigma_{\text{BL6}}^2)(n_{\text{MRL}} + n_{\text{BL6}}) / ((n_{\text{MRL}}n_{\text{BL6}})(n_{\text{MRL}} + n_{\text{BL6}} - 2))}$, where n_{MRL} and n_{BL6} are the number of replicates, μ_{MRL} and μ_{BL6} are the means, and σ_{MRL}^2 and σ_{BL6}^2 are the variances of the expression values for the two

datasets. Probe set mappings to Refseq genes were obtained via Ensembl (www.ensembl.org). Gene descriptions for Refseq genes were obtained via the University of California, Santa Cruz genome browser (genome.ucsc.edu).

For quantitative reverse transcription PCR (qPCR), 10 μ g RNA per sample was treated with RNase-free DNase (Ambion DNA-free; 4 U, 50 μ l, 15 min at 37°C). Two micrograms of DNA-free RNA was reverse transcribed in the presence of random oligonucleotides (Multiscribe High Capacity cDNA Reverse Transcription Kit; Applied Biosystems). qPCR was carried out in triplicate using 20 μ l reactions and Taqman predesigned gene expression assays (Applied Biosystems). cDNA corresponding to 10 ng input RNA was used for each qPCR reaction. Sample data were fitted to standard curves generated from a four-log dilution series of pooled cDNAs. Taqman primer/probe sets for candidate genes were as follows: FGF2, Mm00433287_m1; BDNF, Mm00432069_m1; IGF1, Mm00439561_m1; Sim1, Mm00441390_m1; Ang1, Mm00833184_s1; Galc, Mm00484646_m1; Homer1, Mm00516275_m1; Lhx9, Mm00495310_m1; Pik4ch, Mm00660064_m1; and Stk25, Mm00445502_m1. For each sample, relative expression levels of these genes were normalized to the average expression levels of the housekeeping gene, GAPDH.

Immunohistochemistry

Animals were anesthetized as described earlier and then perfused transcardially with 4% paraformaldehyde in phosphate buffer. The brains were removed, stored in fixative overnight, and transferred into 30% sucrose. Sections (40 μ m thick) were cut coronally on a sliding microtome and stored at -20°C in a cryoprotectant containing 25% ethylene glycol and 25% glycerol in 0.05 M phosphate buffer. Primary and secondary antibodies were diluted in Tris-buffered saline containing 0.1% Triton X-100 and 3% donkey serum.

For BrdU immunohistochemistry, sections were incubated in 50% formamide/50% $2\times$ SSC buffer ($2\times$ SSC is 0.3 M NaCl and 0.03 M sodium citrate, pH 7.0) at 65°C for 2 h, rinsed twice in SSC buffer, incubated in 2 M HCl for 30 min at 37°C , and rinsed in 0.1 M borate buffer pH 8.5 for 10 min. Immunostaining for BrdU and NeuN was done as described previously (Kempermann et al., 1997a). The following primary antibodies were used: rat anti-BrdU ascites (Accurate, Harlan Sera-Lab, Loughborough, England) and mouse anti-NeuN (kindly provided by R.J. Mullen, University of Utah, Salt Lake City, Utah). Secondary antibodies raised in donkey were as follows: anti-rat FITC, anti-mouse Cy3 (Jackson ImmunoResearch, West Grove, PA), and donkey anti-mouse biotinylated antibody (Vector Laboratories, Burlingame, CA). A 1-in-12 series of sections was double-labeled as described earlier and analyzed by confocal microscopy (Zeiss, Bio-Rad, Richmond, CA). One hundred BrdU-positive cells per animal were analyzed for coexpression of BrdU and NeuN to assess the neuronal phenotype, and ratios of cells coexpressing BrdU and NeuN were determined.

Stereology

BrdU-positive cells were counted in a 1-in-6 series of sections (240 μm apart) through a 40 \times objective (Leitz) throughout the rostrocaudal extent of the granule cell layer. A 1-in-6 series of adjacent sections between -1.06 and -3.04 mm from Bregma was stained with Hoechst 33342 (Molecular Probes, Eugene, Oregon; 0.5 mg/ml in Tris-buffered saline for 15 min) and used to measure the granule cell layer volume. The granule cell area was traced using a semiautomatic stereology system (StereoInvestigator, MicroBrightfield) and a 10 \times objective. The granule cell reference volume was determined by multiplying the traced granule cell areas for each section by the distance between sections sampled. The number of BrdU-labeled cells was then related to granule cell layer sectional volume and multiplied by the reference volume to estimate the total number of BrdU-positive cells.

Serial-Section Transmission Electron Microscopy

Ten to 12 h after the last water maze trial, mice were deeply anesthetized as described earlier and perfused using a Ringer's solution followed by a solution containing 4% paraformaldehyde and 0.5% glutaraldehyde. Brains were removed, postfixed in the same fixative for 24 h at 4°C, and sliced at 100 μm thickness using a vibratome. Slices were postfixed again in 3% glutaraldehyde for 24 h at 4°C, followed by osmium tetroxide 2%, dehydration in an ascending series of ethanol concentrations, and finally impregnation in epoxy resin. Blocs were trimmed to include both the dentate gyrus and the CA1. Series of 40–70 serial sections were cut at a thickness of 40 nm, counterstained using uranyl acetate and lead citrate solutions, and observed at a magnification of 19,000 \times using a Jeol C10 electron microscope. Synapses in the CA1 were imaged in the middle third of the stratum radiatum, and synapses in the dentate gyrus were imaged in the outer third of the stratum moleculare. Stereology was performed as previously described (Sterio, 1984). Synapse density was determined by systematic random sampling on 50 dissector pairs in each area, using the postsynaptic density as counting unit.

To analyze the proportion of multiple synapse boutons (MSBs), 80–120 synapses per area were fully reconstructed in three-dimensional space, and the proportion of MSBs over the total number of synapses was calculated. MSBs were defined as the synaptic contact between an axon terminal and more than one dendritic spine. Synapses in which the axon terminal could not be reconstructed in its entirety were omitted from the analysis.

Spatial Learning

Mice were trained on a Morris water maze (Morris, 1984) with three daily blocks of four trials each, for either six blocks (for electron microscopic studies) or 13 blocks (for all other analyses). The platform was visible only during the first block. The starting points changed every trial and each trial lasted until the mouse found the platform, for a maximum of 60 s;

the time to reach the platform (escape latency) was recorded. At the end of each trial, the mice were allowed to rest on the platform for 30 s and were returned to their cages for at least 15 min until the following trial. Twenty-four hours after the last block, we performed a probe trial: the platform was removed and mice were tested for 60 s. The proportion of time spent in the quadrant that previously contained the platform was then calculated.

Visual-Paired Comparison Task

The VPC task has been adapted from previously described protocols (Clark et al., 2000; Korzus et al., 2004). The task is divided into four phases: habituation, familiarization, delay, and test. (1) Mice were handled twice a day during the 3 days preceding the experiment. They were then placed in their home cage in the experimental room for 2 h before the experiment. (2) During familiarization, animals were individually presented with two identical objects in the testing chamber for 30 min. (3) Animals were returned to their home cage for 30 min or for 24 h until the test phase was started. (4) During the test phase, animals were transferred to the testing chamber and, 2 min later, both the familiar object and a novel object were placed in the chamber. Exploration time was recorded for a 5-min test period. The animals' movements were observed via a camera located over the chamber and were recorded on videotape and later analyzed on a large screen. Object exploration (animal's head oriented toward the object and vibrissae moving) was scored for a total of 30 s of exploration time. Objects and chambers were cleaned with 85% ethanol and rinsed with water in between each session and each animal.

Statistical Analysis

Values are expressed as mean \pm SEM. For water maze tasks, we determined the overall significance using repeated measures ANOVA test and post hoc Bonferroni test, using the Statview software. For all other data, we used the Student's *t*-test.

RESULTS

Adult Neurogenesis

Neurogenesis is defined as the birth of new nerve cells and consists of a series of distinct steps, three of which can be examined separately: proliferation, survival, and differentiation. To compare the proliferation of adult neuronal stem cells between MRL/MpJ and C57BL/6 strains, we gave 10-week-old mice daily injections of the proliferation marker, BrdU (50 $\mu\text{g}/\text{g}$ body weight), for 6 days, and we analyzed BrdU incorporation 24 h after the last injection. The dentate gyrus of MRL/MpJ mice contained 60% fewer BrdU-labeled cells than that of C57BL/6 mice ($1,652 \pm 174$ and $4,046 \pm 150$ cells, respectively; $P < 0.0001$; Fig. 1). As in C57BL/6 mice (Lagace et al., 2007), these measures in MRL/MpJ were constant throughout

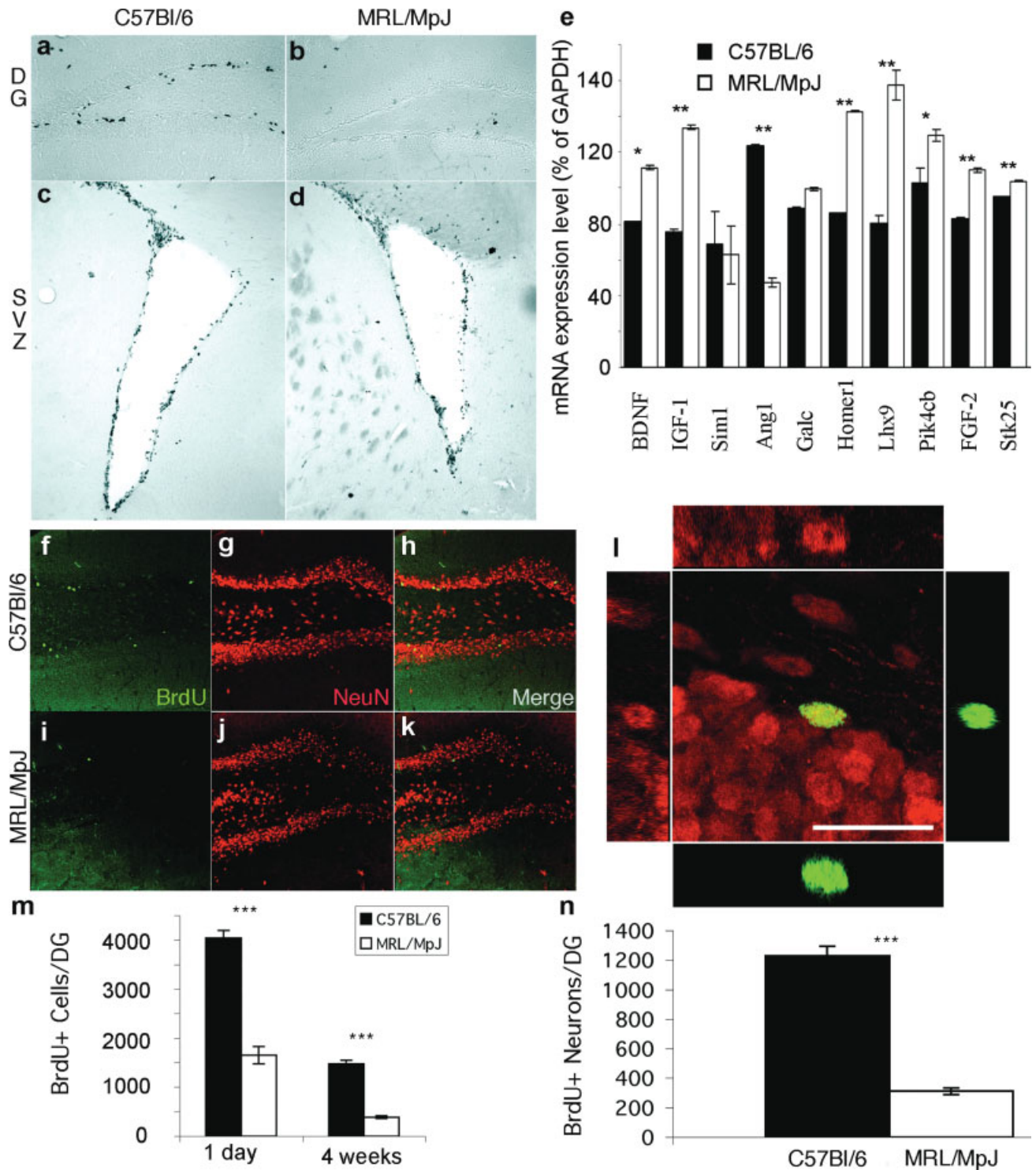


FIGURE 1. Adult neurogenesis. Photomicrographs of BrdU-positive cells in the dentate gyrus (a, b) and in the subventricular zone (SVZ) (c, d) 1 day after the last BrdU injection in C57BL/6 (a, c) and MRL/MpJ (b, d) mice. (e) Difference in mRNA-expression level (analyzed using quantitative PCR) between the two strains. Immunostaining of BrdU-positive cells in the DG 4 weeks after the last injection in C57BL/6 (f–h) and MRL/MpJ (i–k) mice. Sections were double labeled for BrdU (green) and NeuN

(red). Colocalization was identified using confocal microscopy and 3D reconstructions (l). (m) Quantification of BrdU-positive cells in the DG 1 day and 4 weeks after the last BrdU injection. (n) Quantification of cells expressing NeuN relative to the total number of BrdU-positive cells per DG, 4 weeks after the last BrdU injection. *** $P < 0.0001$; ** $P < 0.01$; * $P < 0.05$. Bars: 20 μm (l). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the estrous cycle (Proestus: $1,623 \pm 115$ cells; Estrus: $1,560 \pm 114$ cells; Diestrus: $1,512 \pm 103$ cells; ANOVA Single factor: $F(2, 6) = 0.25$; $P = 0.8$, data not shown). The observed reduction in the numbers of BrdU+ cells in the MRL/MpJ hippocampus may be the result of a generally lower level of BrdU bioavailability in this strain of mice, and not necessarily reflect a hippocampus-specific reduction of cell proliferation. To test this possibility, we examined proliferation in the subventricular zone. We did not detect any difference in the numbers of BrdU+ cells in the subventricular zone between MRL/MpJ and C57BL/6 mice, indicating that the lower levels of cell proliferation in MRL/MpJ mice were restricted to the dentate gyrus (Figs. 1c,d). Additionally, to test the possibility that lower levels of cell proliferation in MRL/MpJ mice were caused by a smaller hippocampus, we used unbiased stereology to measure the volumes of the hippocampus and the granule cell layer and counted the total number of granule cells in both strains. For these parameters, we did not detect any significant differences between MRL/MpJ and C57BL/6 mice (hippocampal volume: $5.59 \pm 0.13 \text{ mm}^3$ vs. $5.41 \pm 0.31 \text{ mm}^3$; granule cell layer volume: $0.29 \pm 0.02 \text{ mm}^3$ vs. $0.26 \pm 0.02 \text{ mm}^3$; total number of granule cells: $282,000 \pm 16,000$ vs. $238,000 \pm 20,000$, respectively; $n = 4$ and $P > 0.1$ for all measures).

In an effort to begin identifying molecular mechanisms that may be involved in the interstrain proliferation differences, we performed an mRNA microarray analysis on the hippocampi of the two strains of mice (see Methods section). At a P -value cutoff of 0.01 and correcting for multiple hypotheses testing, 1,024 (2.3% of all probes) and 374 (0.8%) probes were differentially enriched in the MRL/MpJ and C57BL/6 strains, respectively. Gene ontology analysis revealed significant enrichment in translational initiation ($P < 3 \times 10^{-6}$), intracellular protein transport ($P < 3.2 \times 10^{-5}$), and protein biosynthesis ($P < 2 \times 10^{-4}$) in the MRL/MpJ mice. Therefore, we surmised that there were no large-scale gene expression differences in neuron-related pathways. However, we did identify potential candidate genes whose functions were related to neuronal proliferation, differentiation, or function that changed dramatically. The RNA-expression level of these candidate genes was examined using qPCR (Fig. 1e). A Student's t -test showed significant interstrain differences for many genes related to neural stem cell proliferation, such as brain-derived neurotrophic factor (BDNF), insulin-like growth factor (IGF-1), and fibroblast growth factor (FGF2), but also for kinases, such as the phosphatidylinositol-4-kinase, catalytic, beta (Pik4cb), and the serine-threonine kinase 25 (Stk25), for transcription factors such as the LIM homeobox protein 9 (Lhx9), and for the ribonuclease angiogenin RNase A family 5 (Ang1) and the synaptic protein Homer1.

We next assessed the survival of newborn cells by comparing the number of BrdU-positive cells 4 weeks after the last injection with the number of labeled cells 24 h after the last injection. The total number of BrdU-positive cells after 4 weeks in MRL/MpJ mice was 75% lower than in C57BL/6 mice (389 ± 28 and $1,473 \pm 75$ cells, respectively; Student's t -test, $P < 0.0001$; Figs. 1f–m). When the number of labeled cells after 4 weeks was expressed as a ratio of the number of labeled cells at

24 h after the last injection, we found that the survival rate was 1 cell in 4.3 in MRL/MpJ mice and 1 in 2.7 in C57BL/6 mice; therefore, survival was also reduced in MRL/MpJ mice.

Finally, we examined the differentiation of BrdU-positive cells into neurons 4 weeks after the last injection by colabeling for BrdU and the neuron-specific marker, NeuN (Fig. 1l). In MRL/MpJ, neurons accounted for $80 \pm 0.8\%$ of the surviving BrdU-positive cells when compared with $83.4 \pm 1.9\%$ in C57BL/6 (Student's t -test, $P > 0.1$; data not shown), indicating that differentiation was similar in both strains.

Altogether, these data indicate that adult MRL/MpJ mice generate 75% fewer neurons per dentate gyrus than C57BL/6 mice (311 ± 23 and $1,229 \pm 63$ cells, respectively; Student's t -test, $P < 0.0001$; Fig. 1n). As in C57BL/6 mice (Lagace et al., 2007), these measures in MRL/MpJ were constant throughout the estrous cycle (Proestus: 289 ± 37 cells; Estrus: 378 ± 11 cells; Diestrus: 409 ± 41 cells; ANOVA Single factor: $F(2, 6) = 0.58$; $P = 0.6$, data not shown).

Learning Tests

We tested learning performances in MRL/MpJ mice using spatial memory and object recognition memory tests. To test spatial learning, we used the Morris water-maze task. Ten MRL/MpJ mice and 10 C57BL/6 mice were given four trials per block, three blocks per day for a total of 13 blocks, and the escape latency was recorded. To test the ability to find the platform, the first block was performed using a visible platform, which was then hidden for the next 12 consecutive blocks. Twenty-four hours after the last block, the platform was removed and all mice were subjected to a probe trial. During the first five blocks, no interstrain differences were observed, indicating similar motivation and motor performances. During the entire test, however, C57BL/6 mice reached the platform significantly faster than MRL/MpJ mice (one-way ANOVA, $F(1,18) = 10.62$, $P = 0.0044$; post hoc Bonferroni test $P = 0.006$, Fig. 2a). This difference was not due to physical differences, because swim speed was similar between both strains (MRL/MpJ: $18.2 \pm 0.7 \text{ cm/s}$; C57BL/6: $17.5 \pm 1.9 \text{ cm/s}$; Student's t -test, $P > 0.5$, data not shown). Furthermore, the difference was not due to a platform searching strategy, because during the probe trial all mice stayed longer in the quadrant that contained the platform than in any other quadrant. However, MRL/MpJ mice stayed in the platform quadrant for significantly shorter periods of time than did C57BL/6 mice ($37.7 \pm 1.6\%$ vs. $50.4 \pm 2.8\%$, respectively, Student's t -test, $P < 0.001$, data not illustrated). Thus, spatial learning is impaired in MRL/MpJ mice.

To test recognition memory, we used the VPC task, which exploits a subject's natural preference for novel objects. Ten C57BL/6 and 10 MRL/MpJ mice were first shown two identical objects. We recorded the exploration times and observed that the mice spent an identical amount of time observing both objects, indicating a lack of preference. Thirty minutes later, one of the familiar objects was replaced by a novel object. C57BL/6 and MRL/MpJ mice spent most of their time explor-

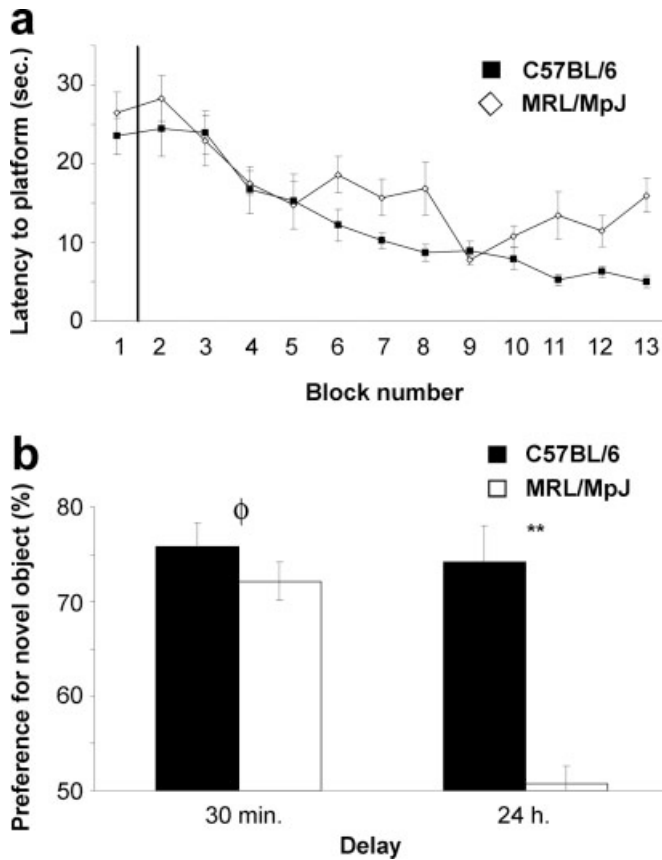


FIGURE 2. Water maze test and visual-paired comparison test. (a) Latency in seconds to reach the platform in the water maze test for C57BL/6 (closed squares) and MRL/MpJ (open lozenges) mice. The platform was visible only during the first block. (b) Preference in percentage for the novel object in the visual-paired comparison test after 30 min and 24 h delay. C57BL/6, black; MRL/MpJ, white. $**P < 0.0001$; $\phi P > 0.2$.

ing the new object ($75.8 \pm 2.5\%$ and $72.1 \pm 2\%$, respectively; Student's *t*-test, $P > 0.2$; Fig. 2b), indicating that short-term recognition memory was similar for both strains. After a 24-h delay, a second novel object replaced the new object that was presented for the 30-min test. C57BL/6 mice spent most of their time exploring the new object, whereas MRL/MpJ mice explored the new object only for about half the time ($74.2 \pm 3.9\%$ and $50.7 \pm 1.9\%$, respectively; Student's *t*-test, $P < 0.0001$; Fig. 2b), indicating that memory of the familiar object was retained for less than 24 h in MRL/MpJ mice. Thus, long-term recognition memory is also impaired in MRL/MpJ mice.

Synaptic Remodeling

One of the mechanisms by which synaptic plasticity plays a role in the formation of new memories involves the learning-induced addition of new synapses on activated presynaptic terminals (Toni et al., 1999, 2001; Geinisman et al., 2001), resulting in the formation of MSB and the strengthening of activated synapses (Fig. 3b) (Sorra and Harris, 1993; Shepherd and Harris, 1998).

To analyze learning-induced synaptic remodeling in MRL/MpJ mice, we compared synapse ultrastructure between learner and nonlearner animals using ssTEM. Three MRL/MpJ mice and five C57BL/6 mice were given three daily blocks of four water maze trials each, over two consecutive days. The first block was conducted using a visible platform. As control, three MRL/MpJ and four C57BL/6 nonlearner mice were housed in standard conditions. Similar to the results shown in Figure 2a, we did not detect any significant difference in the escape latency between groups for the five first blocks (One-way ANOVA $F(1,4) = 0.632$, $P = 0.471$, Fig. 3a), but the time to reach the platform was significantly shorter for block 6 than for block 2 for both strains (post hoc *t*-test $P < 0.05$), indicating that learning had occurred in both C57BL/6 and MRL/MpJ mice. Ten to 12 h after the last trial, all mice were perfused and processed for ssTEM. In C57BL/6 mice, learning induced a significant increase in the proportion of MSB in the dentate gyrus ($36.2 \pm 1.9\%$ in nonlearners, $51.1 \pm 4.9\%$ in learners; Student's *t*-test $P < 0.01$; Fig. 3c), but not in the CA1 hippocampal subfield. In contrast, learning did not induce morphological modifications in the dentate gyrus of MRL/MpJ mice (Fig. 3c). To test whether this observed reduction of synaptic plasticity in MRL/MpJ mice might have resulted from differences in synapse density, we measured synapse density in the CA1 area and the dentate gyrus of both strains using stereology. Synapse density was similar in both strains in both the CA1 and the dentate gyrus and averaged 2.46 synapses/ μm^3 (Fig. 3d). Thus, synapse density was comparable between the two strains, but learning induced morphological modifications in the dentate gyrus of C57BL/6 mice but not in MRL/MpJ mice.

Effect of Voluntary Running on Neurogenesis

Voluntary running increases neural stem cell proliferation, survival, and neuronal differentiation in rodents (van Praag et al., 1999b). To test whether neural stem cells of MRL/MpJ mice retained the capacity to respond to such environmental stimuli, eight MRL/MpJ and nine C57BL/6 mice were individually housed in cages containing a running wheel for 4 weeks and were injected daily with BrdU for the first 6 days. Running activity was recorded on an hourly basis for the entire length of the housing (Fig. 4a). On an average, MRL/MpJ mice ran 13.9 ± 1.0 km per 24 h and C57BL/6 mice ran 8.0 ± 0.6 km per 24 h (Student's *t*-test, $P < 0.001$; data not illustrated). Four weeks after the last injection, the number of BrdU-labeled cells was counted. Running increased BrdU-labeled cells in both MRL/MpJ and C57BL/6 mice. Surprisingly, the extent of this increase was such that the number of BrdU-labeled cells was now comparable between both strains (MRL/MpJ: 3096.1 ± 55.8 , and C57BL/6: $3,448 \pm 509.5$ cells per dentate gyrus, Student's *t*-test, $P > 0.1$; Figs. 4b–h). Therefore, running induced a 790% increase in BrdU incorporation in MRL/MpJ mice, compared with a 230% increase in C57BL/6 mice.

We then stained for the neuronal marker, NeuN, and counted the proportion of BrdU-labeled cells that colabeled with NeuN. In MRL/MpJ mice, neurons accounted for

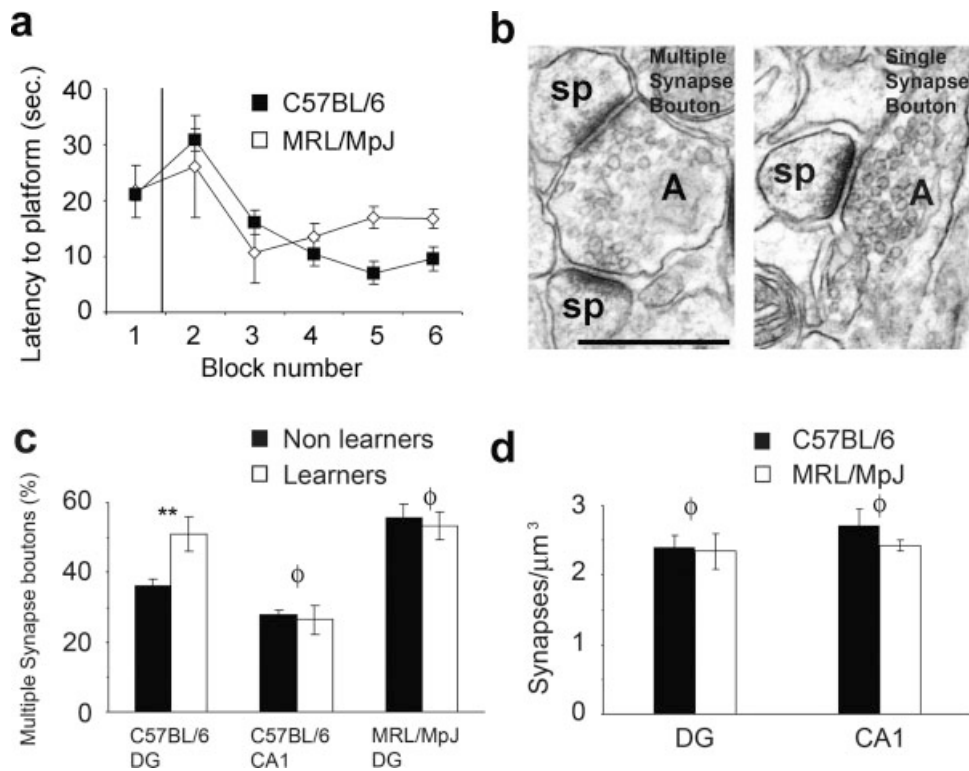


FIGURE 3. Synapse ultrastructure and remodeling after spatial training. (a) Latency in seconds to reach the platform in the water maze test for the mice used for ssTEM analyses. (b) Electron micrograph of a multiple-synapse bouton (MSB, left) and a single-synapse bouton (SSB, right) showing axon terminals (A) making synaptic connections with dendritic spines (sp). Scale bar, 0.5 μm .

86.7 \pm 1.4% of the surviving BrdU-positive cells and for 88.3 \pm 0.8% in C57BL/6. Thus, voluntary running significantly increased neurogenesis in both strains (Student's *t*-test, $P < 0.01$), but there was no significant difference between the two running groups (Student's *t*-test, $P > 0.1$; Fig. 4i). Altogether, MRL/MpJ runners generated on average a total of 2684.7 \pm 168.8 BrdU-labeled neurons per dentate gyrus vs. 3044.5 \pm 159 in C57BL/6 running mice during the 6 days of BrdU injection (Student's *t*-test, $P > 0.1$; Fig. 4j). Thus, voluntary running completely rescued neurogenesis in MRL/MpJ mice.

Effect of Voluntary Running on Spatial Learning and Recognition Memory

Nine MRL/MpJ mice and eight C57BL/6 mice were housed for 4 weeks in individual cages with a running wheel and tested thereafter in the water maze using the same parameters as for Figure 2. When all four groups (runners, nonrunners, C57BL/6, MRL/MpJ) were compared using a two-way ANOVA test with repeated measures, we found that exposure to running had a different effect on the strains ($F(1,33) = 11.7$; $P = 0.02$). Running significantly reduced the time to reach the platform for MRL/MpJ mice (One-way ANOVA, $F(1,17) = 21.7$; $P = 0.0003$; post hoc Bonferroni test, $P = 0.001$, Fig. 5b) but not for C57BL/6 mice (One-way ANOVA, $F(1,16) = 2.6$;

(c) Proportion of dendritic spines contacting MSB in the dentate gyrus and in the CA1, under control conditions (nonlearners) and after water maze training (Learners) for C57BL/6 and MRL/MpJ mice. (d) Synapse density in the dentate gyrus and the CA1 area of both C57BL/6 and MRL/MpJ mice. ** $P < 0.01$; $\phi P > 0.05$.

$P = 0.1$; Fig. 5a). Surprisingly, the time to reach the platform was significantly shorter for MRL/MpJ mice than for C57BL/6 mice (One-way ANOVA, $F(1,15) = 24.6$; $P = 0.0002$, post hoc Bonferroni test, $P = 0.045$, Fig. 5c). Twenty-four hours after the last trial, the platform was removed and all runner mice were subjected to a probe trial. Both strains spent a similar proportion of their time in the quadrant that previously contained the platform (MRL/MpJ: 32.9 \pm 1.3%, C57BL/6: 37.2 \pm 2.1%, Student's *t*-test, $P > 0.05$, data not illustrated).

Thus, after voluntary running, MRL/MpJ mice performed as well or better than C57BL/6 mice on the water maze task.

The VPC test was performed following the same protocol described in Figure 2, using the same set of objects in the same conditions and environment. During the familiarization phase, the mice spent an equal amount of time exploring both identical objects, indicating a lack of preference. After a delay of 30 min, one object was changed and C57BL/6 and MRL/MpJ spent 72.1 \pm 5.2% and 74.4 \pm 4.2%, respectively, of their time exploring the new object. Thus, there was no significant difference in exploration time between the two strains (Student's *t*-test, $P > 0.3$; Fig. 5d). After a delay of 24 h, the new object was replaced again and the C57BL/6 mice spent most (70.1 \pm 4.7%) of their time exploring the new object, whereas MRL/MpJ mice explored the new object at chance (54.4 \pm 10.2%, Student's *t*-test, $P < 0.002$; Fig. 5d). Thus, the deficit

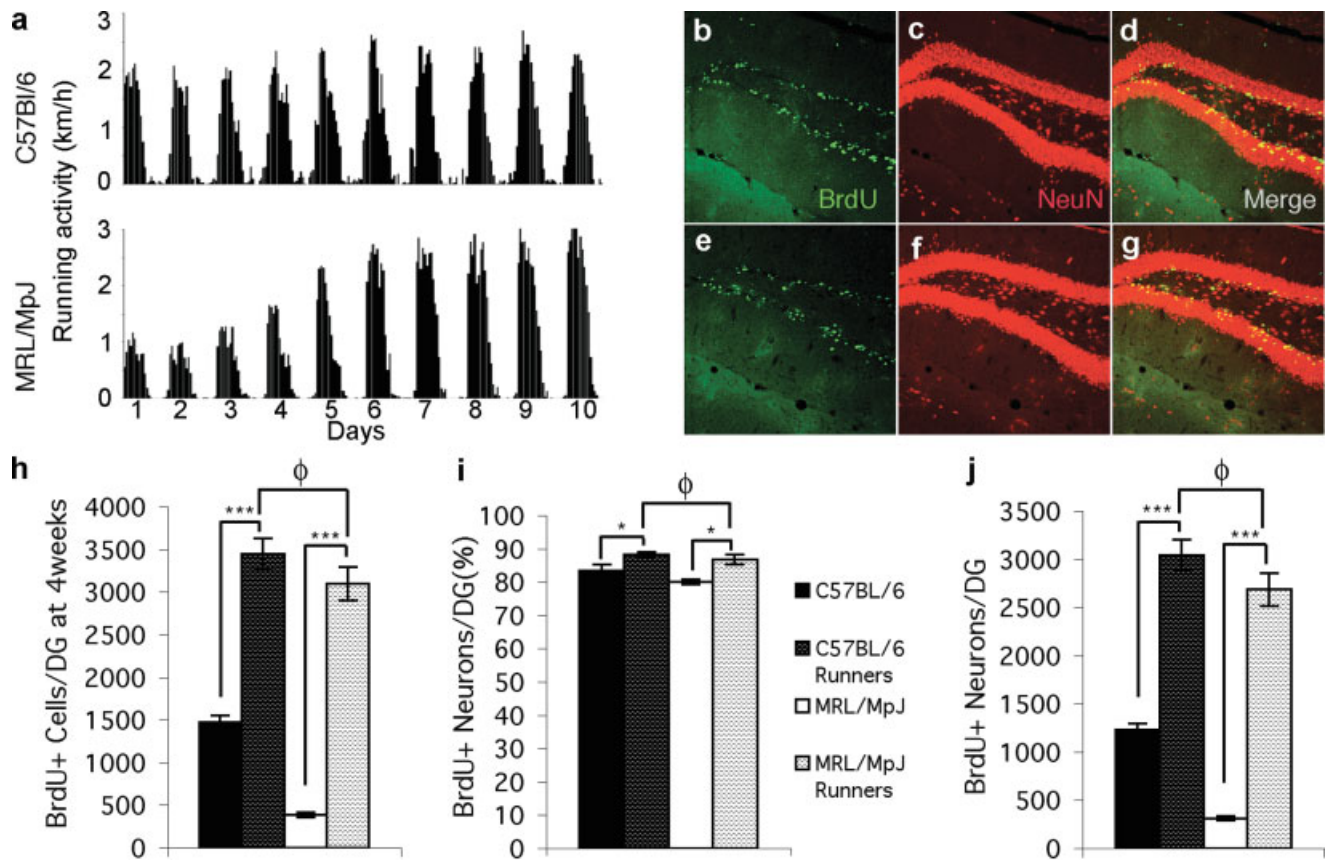


FIGURE 4. Adult neurogenesis in the dentate gyrus after voluntary running. (a) Average running activity for both strains for the first 10 days of housing in a cage containing a running wheel. Photomicrographs of BrdU-positive cells in the DG 4 weeks after the last injection in C57BL/6 (b–d) and MRL/MpJ (e–g) running mice. Sections were double labeled for BrdU (green) and NeuN (red). (h) Quantification of BrdU-positive cells in the DG 4 weeks

after the last BrdU injection. (i, j) Quantification of BrdU-positive cells expressing NeuN in percentage of BrdU-positive cells per DG (i), and relative to the total number of BrdU-positive cells per DG, 4 weeks after the last BrdU injection (j). *** $P < 0.0001$; * $P < 0.01$; $\phi P > 0.1$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

of MRL/MpJ mice in long-term recognition memory was not rescued by 4 weeks of running. Hypothesizing that the duration of voluntary running was too short to produce a detectable effect on the VPC test, we returned all mice to their original running wheel cages for 7 additional weeks, after which we performed another VPC test using a new set of objects. At this later time point, after a 24-h delay, MRL/MpJ mice spent as much time as the C57BL/6 mice exploring the new object ($70.1 \pm 2.9\%$ and $72.0 \pm 2.9\%$, respectively, Student's t -test, $P > 0.3$; Fig. 5d). Therefore, the deficit in spatial learning for MRL/MpJ mice was rescued after 4 weeks of voluntary running, and the deficit in visual recognition memory was rescued only after 11 weeks of running.

DISCUSSION

In this study, we report that MRL/MpJ mice have a low level of neurogenesis, which was associated with undetectable learning-induced synaptic remodeling and poor performances

in hippocampus-dependent learning tasks when compared with C57BL/6 mice. We show that, after running, neurogenesis was restored in MRL/MpJ mice and their learning deficit was rescued. These findings suggest that neurogenesis is correlated with hippocampus-dependent learning in MRL/MpJ mice and that voluntary running can abolish interstrain differences in neurogenesis.

In this study, we test the function of adult neurogenesis by assessing a naturally occurring mutant strain that shows a dramatic reduction in neurogenesis. These results support the previous observation that adult neurogenesis plays a role in spatial long-term memory (Snyder et al., 2005), and they extend these observations to another form of hippocampus-dependent memory, the visual recognition task. The number of granule cells, the granule cell layer volume, the hippocampal volume, and stem cell proliferation in the subventricular zone are similar between the strains, indicating that MRL/MpJ mice do not suffer from general downregulation of stem cell/progenitor cell function in the brain but rather that neurogenesis is selectively reduced in the adult dentate gyrus. Furthermore, synapse density is similar, both in the CA1 and the dentate gyrus (Fig. 3),

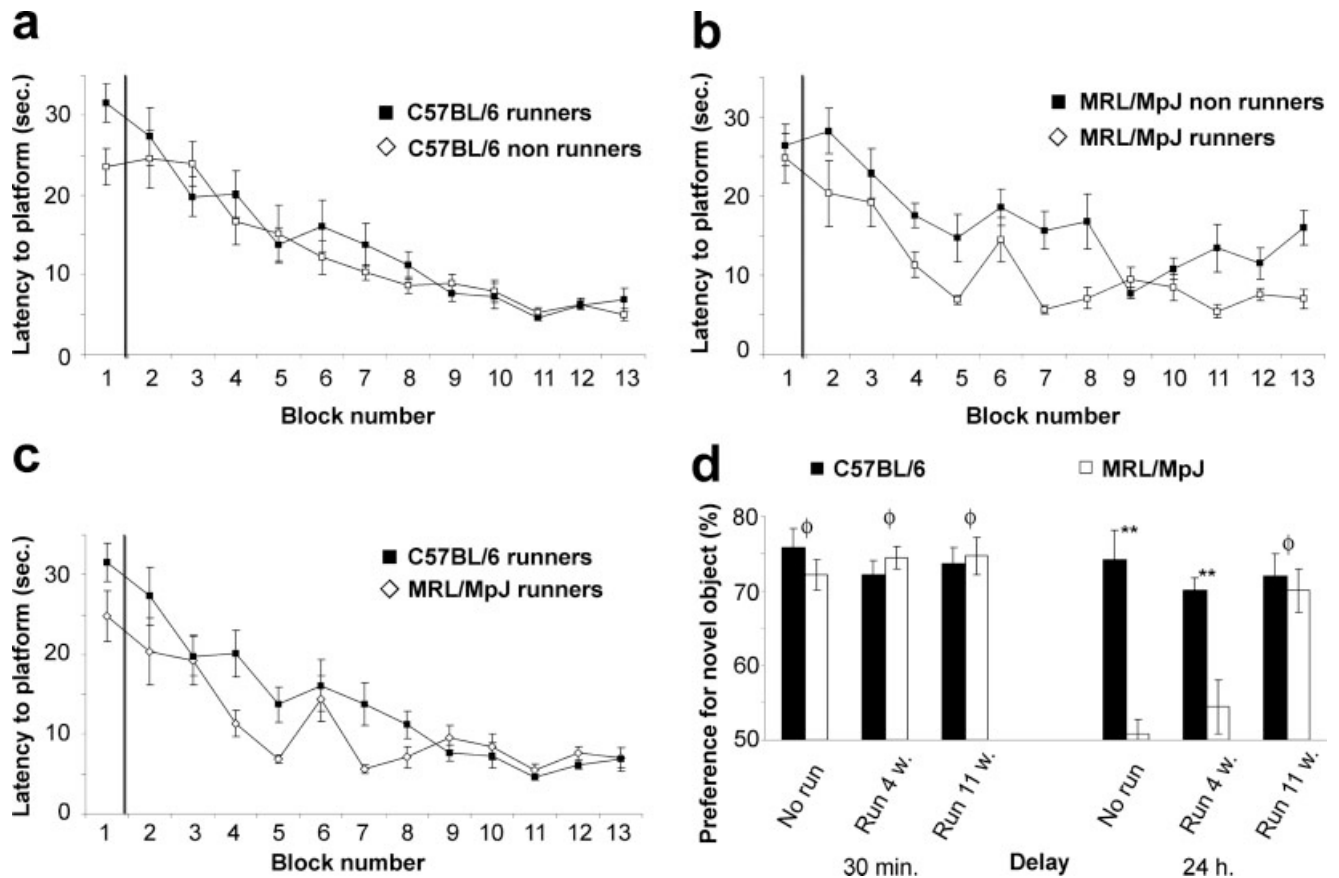


FIGURE 5. Water maze test and visual-paired comparison test after voluntary running. (a–c) Latency to reach the platform in the water maze test for C57BL/6 runners and nonrunners (a), MRL/MpJ runners and nonrunners (b), and C57BL/6 runners and MRL/MpJ runners (c). The platform was visible during the first

block only. (d) Preference for the novel object in the visual-paired comparison test after 30-min and 24-h delays for both C57BL/6 (black columns) and MRL/MpJ (white columns) mice housed without running wheels and after either 4 or 11 weeks of housing in a running wheel cage. $**P < 0.002$; $\phi P > 0.3$.

indicating that synaptogenesis itself is not altered simply as a result of reduced neurogenesis in MRL/MpJ mice. Finally, visual acuity and short-term recognition memory are not affected in MRL/MpJ mice, because VPC tests yielded comparable results after a 30-min delay (Fig. 2). Thus, MRL/MpJ mice seem perfectly suited for the behavioral and morphological tests that we performed in this study and represent a useful model for studying the effect of reduced adult neurogenesis on hippocampal function. However, we cannot rule out the possibility that the reduction of synaptic plasticity and learning in MRL/MpJ mice is due to mechanisms other than decreased neurogenesis, such as the ovarian cycle or gene expression. Indeed, the RNA-expression analyses uncover a list of genes that are differentially expressed in the hippocampus of the two strains of mice (Fig. 1). Some of these genes are known regulators of adult neurogenesis (BDNF, FGF-2, IGF-1), whereas some others are involved in neuronal differentiation (Lhx9), synapse formation and plasticity (Homer1), vascularization (Ang1), second messenger formation (Pik4cb), or stress-related response (stk25). The variety of genes whose mRNA is differentially expressed in the hippocampus of MRL/MpJ and C57BL/6

mice suggests that the learning differences may depend on several regulatory mechanisms.

Adult Neurogenesis

We observed that MRL/MpJ mice produce 75% fewer new neurons than C57BL/6 mice. This reduction is constant throughout the estrous cycle, surpasses the interstrain differences that were previously reported (Kempermann et al., 1997a) and lies within the range of the reduction obtained by treatment with the antimitotic drug, methylazoxymethanol acetate (Shors et al., 2001).

Voluntary running increased neurogenesis in both MRL/MpJ and C57BL/6 mice, supporting our previous observation of the effect of exercise on adult neurogenesis (van Praag et al., 1999b). It is, however, remarkable that the increase is greater in MRL/MpJ than in C57BL/6 mice and results in the elimination of differences in neurogenesis between these strains. Thus, the deficit in neurogenesis of MRL/MpJ mice can be completely overcome with voluntary running. This finding indicates that the capacity of neural stem/progenitor cells to

respond to environmental stimuli is retained in MRL/MpJ mice. What accounts for the increased effect of running on MRL/MpJ mice?

One possibility is that inactive progenitors are more responsive to stimulation. This possibility is supported by the observation of neurogenesis in the aged brain. Neurogenesis decreases with aging, but environmental enrichment induces a threefold increase in neurogenesis in the aged brain and only a twofold increase in the younger brain (Kempermann et al., 1998, 2002; van Praag et al., 2005). This finding suggests that extrinsic factors play an important role in the inhibition of neurogenesis in the adult MRL/MpJ hippocampus.

Another possibility is greater distances run, because neurogenesis was found to be linearly correlated with the amount of running up to 6 km/day in outbred mice but not in mice bred for increased running (Rhodes et al., 2003). In support of this possibility, we found that MRL/MpJ mice ran, on average, 60% greater distances than C57BL/6 mice, although their circadian rhythm was similar. Combined with the behavioral experiments of Figure 5, our results suggest that distance run also influences learning performances. Indeed, spatial learning performances were improved in good runner mice only (MRL/MpJ), and increasing exposure to running wheels also increased the learning performances on the visual learning task.

Synaptic Remodeling

MSBs result from the synaptogenesis induced by long-term potentiation (LTP) (Toni et al., 1999) or associative learning (Geinisman et al., 2001). This form of synaptic plasticity is believed to play a role in the late phases of learning and to strengthen synaptic transmission between activated neurons (Lamprecht and LeDoux, 2004).

Our observation that, in C57BL/6 mice, spatial learning increased the proportion of MSBs in the dentate gyrus but not in the CA1 suggests that, under our experimental conditions, the dentate gyrus was more involved in spatial learning than the CA1 area. This finding, together with the absence of learning-induced MSB formation in MRL/MpJ mice, indicates that structural plasticity is correlated with adult neurogenesis, suggesting that the function of new neurons may be mediated by an increase in synaptic remodeling. This possibility is supported by the recent report that, in the dentate gyrus, immature neurons integrate into the hippocampal circuitry (Toni et al., 2007) and express LTP more easily than mature neurons (Farmer et al., 2004; Schmidt-Hieber et al., 2004; Ge et al., 2007).

Spatial Learning

In standard housing, we observed that the performance of MRL/MpJ mice in spatial learning was lower than that of C57BL/6 mice, supporting our previous observation that the amount of neurogenesis predicts performance on the water maze task in different mouse strains (Kempermann and Gage, 2002).

We further observed that running MRL/MpJ mice had better performances than nonrunners in the water maze test, supporting previous reports that enriched environment and voluntary exercise increase spatial learning in healthy mice (Kempermann et al., 1997b; van Praag et al., 1999a,b) and rescue learning deficits associated with ethanol exposure (Crews et al., 2004; Christie et al., 2005), stroke (Luo et al., 2007), or aging (van Praag et al., 2005). We observed, however, that, after 4 weeks of running, learning did not improve in C57BL/6 mice. The discrepancy between our results and our previous report (van Praag et al., 1999a) is very likely because of a difference in the paradigm used for the water maze test. In this study, mice were presented with 12 trials per day, whereas in the previous study, mice were exposed to the water maze for only four trials a day, which is probably more challenging.

The observation that running improved learning in MRL/MpJ but not in C57BL/6 mice suggests that spatial learning may depend on the magnitude of the increase in neurogenesis rather than on the total number of new neurons (running increased neurogenesis by 2.3-fold for C57BL/6 and of 7.9-fold for MRL/MpJ mice), which may itself depend on distance run by the mice (MRL/MpJ mice ran in average 170% the distance covered by C57BL/6). Alternatively, MRL/MpJ mice may use compensatory mechanisms to cope with the reduction of neurogenesis, such as a modified connectivity of granule neurons, as seen in the high proportion of MSBs (Fig. 3c), or overexpression of genes regulating stem cell proliferation (Fig. 1e). The running-induced neurogenesis may combine with these mechanisms to produce the increased effect on learning. Although our study cannot provide a definite answer to this question, it supports the idea that adult neurogenesis is associated with spatial learning.

Object Recognition Memory

For the VPC task, the performance of MRL/MpJ and C57BL/6 mice at the 30-min delay was identical and both mice recognized the familiar object. At 24-h delay, MRL/MpJ mice exploration was at chance, whereas C57BL/6 mice still remembered the familiar object. This finding indicates a deficit in long-term recognition memory in MRL/MpJ mice and suggests that neurogenesis may be involved in long-term recognition memory.

Normal hippocampal function is required for performance on the VPC task, and several reports indicate that hippocampal lesions impair recognition memory (Wood et al., 1993; Clark et al., 2000; Zola et al., 2000). Therefore, in light of our water maze results, we were expecting that 4 weeks of voluntary running would also rescue the performance of MRL/MpJ mice in the VPC test. The observation that 11 weeks of running are required to completely rescue VPC task performances, whereas just 4 weeks of running rescue water maze performances, suggests that different mechanisms are involved in the completion of these tasks. One possibility is that the VPC task is more demanding and requires the incorporation of more new neurons than the water maze task. Eleven weeks of running most

likely results in an increase in neurogenesis that is greater than after 4 weeks. This difference may be sufficient to overcome the visual learning deficit. In support of this possibility, we found that 4 weeks of running resulted in a small (but not significant) increase in VPC learning in MRL/MpJ (Fig. 5d). Alternatively or maybe additionally, the VPC task may depend on the maturation stage of new neurons. The VPC task depends on CA1 area function, as indicated by the observations of impaired performance on the VPC task in mice lacking the NMDA α 1 subunit in the CA1 area (Rampon et al., 2000) or after lesions of the CA1 area (Wood et al., 1993). Between 4 and 11 weeks after cell division, new neurons may establish more extended connections and recruit other hippocampal subfields. Indeed, our recent study shows that new neurons attain a mature connectivity only after 4–8 weeks of maturation, during which dendritic spines contact perforant path synapses and axons are projected in the CA3 area (Zhao et al., 2006; Toni et al., 2007, 2008). The improved connectivity resulting from prolonged maturation of new neurons may therefore result in the involvement of the CA1 area that is necessary to perform the VPC task.

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