

Exercise Improves Memory Acquisition and Retrieval in the Y-Maze Task: Relationship With Hippocampal Neurogenesis

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Enhanced physical activity is associated with improvements in cognitive function in rodents as well as in humans. The authors examined in detail which aspects of learning and memory are influenced by exercise, using a spatial Y-maze test combined with a 14-day exercise paradigm at different stages of learning. The authors show that 14 days of wheel running promotes memory acquisition, memory retention, and reversal learning. The exercise paradigm that was employed also significantly increased the number of maturing neurons, suggesting that an increase in neurogenesis underlies the positive effects of exercise on Y-maze performance. Finally, the authors show that memory acquisition in itself does not have a major impact on the number of immature neurons. However, memory retention testing and reversal learning both cause a significant reduction in the number of doublecortin and Ser133-phosphorylated pCREB-positive cells, indicating that a decrease in neurogenesis might be a prerequisite for optimal memory retrieval.

Keywords: retention, reversal learning, doublecortin, pCREB, plasticity

In humans, an active lifestyle and cardiovascular fitness are associated with better cognitive function during aging (Colcombe et al., 2004; Fabre, Chamari, Mucci, Masse-Biron, & Prefaut, 2002). Similarly, enhanced physical activity in laboratory mice and rats has been reported to facilitate memory acquisition and retention in various behavioral tasks, which test different forms of learning. For instance, after 1 month of voluntary running, mice mastered the Morris water maze task faster than sedentary controls did (Van Praag, Christie, Sejnowski, & Gage, 1999; Van Praag, Shubert, Zhao, & Gage, 2005). Rats that had been housed with a running wheel for 4–8 weeks showed increased freezing behavior in the contextual fear conditioning task (Burghardt, Pasumarthi, Wilson, & Fadel, 2006), and exercise also significantly improved retention in the passive shock avoidance task in mice (Samorajski et al., 1985). However, it remains elusive which aspects of the learning process are specifically promoted by exercise.

Therefore, we examined the influence of increased physical activity on learning and memory in more detail. The learning task that we applied is the Y maze, a spatial reference memory task in which the animal has to learn which of two arms is baited with a

food reward. We discriminated between the effects of exercise on memory acquisition and on long-term memory retention. We also assessed the consequences of physical activity on reversal learning, that is, the ability to adapt the acquired information to a new situation and to inhibit the previously involved response (behavioral inhibition).

We chose a left–right discrimination paradigm in the Y maze, instead of the more often applied Morris water maze. The water maze paradigm involves handling and swim stress and causes a considerable elevation of plasma corticosterone levels (Beiko, Lander, Hampson, Boon, & Cain, 2004). Running-wheel activity influences the hypothalamic–pituitary–adrenal axis reactivity by increasing baseline levels of corticosterone but reducing the stress response to novelty (Droste, Schweizer, Ulbricht, & Reul, 2006; Fediuc, Campbell, & Riddell, 2006) and might therefore also alter the stress response to water maze learning. Because we were primarily interested in the cognitive and not the neuroendocrine changes induced by running-wheel activity, we tried to design our experiment in such a way that stress levels are minimized. Although Y-maze learning can also be stressful to the mice because of novelty (Marquez, Nadal, & Armario, 2005), we reduced stress associated with this task by habituating the mice to the maze and by letting them voluntarily enter the apparatus.

In order to distinguish between the effects of exercise on memory acquisition on the one hand and memory retention and reversal learning on the other, we housed mice with a running wheel, either for 14 days prior to acquisition or starting directly after the mice had mastered the task and ending directly before retention testing or reversal learning. In the memory retention task, the food reward was placed in the same arm as during training. Reversal learning was tested by placing the food in the arm opposite to the one that was baited during training.

One of the potential mechanisms that could underlie the exercise-induced improvement in cognition is the increase in neu-

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rogenesis caused by voluntary exercise (Van der Borght et al., 2006; Van Praag, Kempermann, & Gage, 1999). Although the exact function of newborn hippocampal granule neurons has not been elucidated yet, accumulating evidence suggests that they play a role in learning and memory. Hippocampus-dependent learning, for instance, can promote the survival of newborn neurons (Ambrogini et al., 2000; Gould, Beylin, Tanapat, Reeves, & Shors, 1999), although there is some controversy in this field (Snyder, Hong, McDonald, & Wojtowicz, 2005; Van der Borght, Wallinga, Luiten, Eggen, & Van der Zee, 2005; Van Praag, Kempermann, et al., 1999). Furthermore, inhibition of neurogenesis by cytostatic drugs or brain irradiation impairs performance in trace conditioning tasks (Shors et al., 2001; Shors, Townsend, Zhao, Kozorovitskiy, & Gould, 2002) and negatively affects long-term memory in the Morris water maze (Snyder et al., 2005).

In the present study, we examined the potential relationship between exercise, learning and memory, and hippocampal neurogenesis. We hypothesized that runners would not only master the Y-maze task faster than sedentary controls, but that they would also outperform the controls in the memory retention task and the reversal learning paradigm. Our results show that exercise enhanced neurogenesis under restricted food availability. Memory acquisition and memory retention and reversal learning in the Y-maze task were all improved by exercise. Memory retrieval itself induced a reduction in the number of maturing neurons, irrespective of physical activity. Taken together, these results suggest that learning and memory and neurogenesis are related to each other, but that this interaction is complex and highly dynamic.

Materials and Methods

Subjects and Housing Conditions

Eighty 10-week-old male C57BL/6 mice (Harlan, Horst, The Netherlands; 25.8 ± 0.2 g at the beginning of the experiment) were individually housed in standard Macrolon cages ($25 \times 40 \times 25$ cm) equipped with a removable slot that could be locked on to a Y maze, had free access to drinking water, and were kept under a 12-hr light–dark cycle (lights on at 0800). Throughout the entire experiment, all mice, including naive controls, were food restricted to about 85% of their original body weight, which means that they received on average 3 to 4 g of food per day. Mice were fed and weighed daily between 3 p.m. and 5 p.m., after Y-maze training. Food restriction started 1 day prior to the start of the experiment. All procedures concerning animal care and treatment were approved by the ethical committee for the use of experimental animals of the University of Groningen (DEC 4089A and 4089B).

Y-Maze Training Procedure

During behavioral testing, all mice were kept in a standard cage. Behavioral testing was conducted in an enclosed Plexiglas Y maze. The home cage of each mouse was provided with a small sliding door that could be connected to the maze. Both the stem arm (27.5 cm long) and the two arms forming the Y (both 27.5 cm long and diverging at a 60° angle from the stem arm) were 5 cm in diameter. Perforations at the endings of the two arms forming the Y allowed odors from food (standard lab chow, Hope Farms, Woerden, The Netherlands) placed under the perforations to enter both arms.

Small plastic blocks (1 cm high) were placed 4 cm from the endings of the arms to prevent visual inspection for food presence from a distance. Each arm was equipped with a trapdoor halfway into the arm that could be operated manually from the experimenter's position.

In the Y-maze paradigm used in this study, mice had to learn which of the two arms forming the Y was baited with food. The day prior to the start of the training, mice were allowed to freely explore the maze for 5 min. Next, they received two trials, one in which the food was located in the left arm and one in which the food was positioned in the right arm. This procedure prevented the development of a preference for one of the arms. During the training procedure, only one of the two arms contained a food crumb. For half of the mice this was the left arm, and for the other half this was the right arm. In order to avoid any stress-related interference with the learning procedure, mice were not handled by the experimenter but were allowed to voluntarily enter the maze. Whenever a mouse visited one of the two arms, the trapdoor of the nonvisited arm was closed. The mouse was allowed to eat the small piece of food, and after the mouse had reentered its home cage, the arm connected to the home cage was closed. After thorough cleaning of the arms, the mouse was allowed to enter the maze again for the next trial.

Effect of 14 Days of Exercise in Combination With Food Restriction on Hippocampal Neurogenesis

Eight mice were housed with a running wheel made of plastic (diameter 13 cm, width 7 cm; manufactured in our own facilities) for 14 days. The wheels had evenly spaced rods that were covered with a plastic wire mesh (2-mm holes), in order to facilitate running. Sedentary mice ($n = 6$) were housed under standard conditions. Standard cages contained nesting material. The axis of the running wheel, made of stainless steel, was connected to the cage, which contained a sensor that detected revolutions of the wheel. Running-wheel activity was recorded and analyzed with an event recording system (ERS system, Haren, The Netherlands), which stored wheel revolutions in 2-min intervals. Throughout the exercise procedure, mice were subjected to the food restriction paradigm as described above. After the exercise period, mice were sacrificed and brains were processed for immunocytochemistry.

Effect of Physical Exercise on Memory Formation

Mice ($n = 16$) were housed with a running wheel for 14 days. Sedentary mice ($n = 16$) were kept in a standard cage during this period. After the exercise period, all mice were placed in a clean, standard cage in order to avoid extreme variations in sympathetic nervous system activation (Minami et al., 2006), such as increased heart rate and blood pressure, due to running between runners and sedentary mice, which could influence learning speed. One day later 8 exercise mice and 8 sedentary mice were habituated to the Y maze. Training took place on the following 4 days and consisted of two sessions per day, each session containing six trials. The other half of the exercise mice and the sedentary mice were kept in the home cage during the training period and served as naive controls for the Y maze. Mice were sacrificed 1 day after the last training session.

Effect of Physical Activity on Memory Retention and Reversal Learning

Mice ($n = 34$) were trained in the Y maze for 3 days, with two sessions per day. Directly after the last training session, 16 mice were housed with a running wheel for 14 days, and the rest of the mice remained in a standard cage during this period. Groups were matched according to their performance during the last two sessions of the acquisition. One day after the end of the exercise period, all mice were placed in a clean, standard cage for 1 day. The next day, memory retention was tested in half of the exercise mice and half of sedentary mice. These mice were placed again in the Y maze, with the food located in the same arm as it had been during training. The other half of the mice was tested in a reversal learning task, in which the food was located in the arm that was not baited during the training sessions. Mice were exposed to the retention or reversal learning paradigm for two sessions per day for 4 days and were sacrificed 1 day later.

Brain Processing and Immunocytochemistry

Mice were transcardially perfused with heparinized saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed, kept in 0.01 M phosphate-buffered saline overnight, and subsequently cryoprotected in 30% sucrose for 48 hr. Next, 30- μm sections, spanning the dorsal hippocampus (bregma -1.46 to -2.80), were cut on a cryostat microtome. Brains were stained for Ki-67, doublecortin (DCX), and Ser133-phosphorylated CREB (pCREB). Sections were treated with 0.3% H_2O_2 , blocked with 3% normal serum, and the cell membrane was permeabilized with 0.1% Triton-X100. Rabbit-anti-Ki-67 (1:400, Novocastra, Newcastle upon Tyne, UK), goat-anti-DCX (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit-anti-pCREB (1:300, Cell Signaling Technology, Beverly, MA) were applied for 72 hr at 4 °C. Sections were incubated with a biotinylated secondary antibody (rabbit-anti-goat or goat-anti-rabbit, 1:400, Jackson Immunolabs, West Grove, PA) for 2 hr at room temperature, followed by incubation with Avidin-Biotin-Complex (1:400, ABC Elite kit, Vector Laboratories, Burlingame, CA) for 2 hr. Staining was visualized with 20 mg/100 ml 3,3'-diaminobenzidine and 0.03% H_2O_2 .

For the DCX/pCREB double-labeling procedure, a pCREB staining was performed as described above, but without the use of normal serum. The staining was visualized with 3,3'-diaminobenzidine (20 mg/100 ml), nickelammoniumsulfate (200 mg/100 ml), and 0.03% H_2O_2 . Subsequently, sections were treated with a high dose of H_2O_2 (1%), in order to remove all horseradish peroxidase reactivity. Then, sections were stained for DCX, as described above. The DCX-positive cells were visualized with diaminobenzidine (15 mg/100 ml) and 0.01% H_2O_2 .

Quantification of the Immunostainings

The Ki-67 staining was analyzed in every sixth section of the dorsal hippocampus. Immunopositive cells were counted in the subgranular layer with the 40 \times lens. Ki-67 immunoreactive cells that were located one cell diameter deviating from this region were also included in the analysis. Cells were counted throughout the entire thickness of the section, and the total number of counted

cells was multiplied by six to get an estimation of the total number of Ki-67-positive cells per dentate gyrus (DG).

For the analysis of the DCX and pCREB-positive cell number, four sections containing the DG were selected per mouse. Sections were derived from a one-in-six series from the hippocampus, excluding the most rostral and caudal sections. The selected sections were highly comparable between the different mice with respect to their rostrocaudal location. Cells were counted throughout the entire thickness of the section, using a 40 \times magnification. In order to prevent the inclusion of cell profiles in the DCX analysis, we included only cells with a cell soma that was larger than 8 μm in diameter. The inner and outer blades of the granule cell layer were counted separately. For every mouse, the average number of immunopositive cells/section was calculated.

A second measure of the DCX immunostaining was taken in order to verify the DCX cell count analysis. Because most DCX-immunopositive cells possess only one primary dendrite that projects through the granule cell layer, we determined the density of DCX-immunoreactive dendrites in the granule cell layer as a measure for the number of DCX-positive cells. For the density measurements, the same sections were used as for the cell counts. With a computerized system (Leica Qwin, Rijswijk, The Netherlands), two equally sized areas of the inner blade of the granular cell layer and two areas of the outer blade of each hippocampus were delineated. Within the demarcated areas, the percentage of the total surface that was covered with immunopositive dendrites was calculated. Measurements were performed in one focal plane. For every mouse, an average area percentage was calculated from the different measurements.

Statistics

The learning curves of the Y maze were analyzed using a repeated measures analysis of variance (ANOVA). Potential differences in DCX and pCREB immunoreactivity after 14 days of exercise were statistically tested with an independent-samples t test. For the statistical analysis of the Ki-67 staining, the DCX measurements, and the pCREB data of the different Y-maze groups, a two-way ANOVA was used, with exercise and Y-maze training as between-subjects variables. Whenever this revealed a significant difference, multiple comparisons were made with a post hoc Tukey's honestly significant difference test. Data are expressed as averages \pm the standard error of the mean.

Results

Running-Wheel Activity in Combination With Dietary Restriction Promotes Hippocampal Neurogenesis

This study aimed to look at the effects of exercise on various aspects of Y-maze learning and hippocampal neurogenesis. Running-wheel activity has been shown to promote this neurogenesis. However, in the experiments reported here, exercise was combined with a food restriction paradigm. We examined whether neurogenesis is still enhanced by exercise under conditions of reduced food intake.

Mice were housed with a running wheel for 14 days and perfused 1 day later. An overview of the experimental proce-

cedure that was used is presented in Figure 1A. Although cell proliferation (Ki-67 staining) was similar for both groups (see Figure 1B), we show that 14 days of exercise significantly increased the number of pCREB-positive cells in the DG ($p \leq .01$; see Figure 1C), and this was observed in both blades of the granule cell layer (separate data sets not shown). DCX immunocytochemistry showed that the number of immature neurons was also significantly increased by the exercise procedure (see Figure 1D; $p \leq .01$). This increase was visible in both the inner and the outer blade of the granule cell layer (separate data sets not shown). The difference between runners and control mice was confirmed by the DCX-positive dendrite density measurements (see Figure 1E; $p \leq .001$). Over the 14-day exercise period, runners ran 151 ± 8 km. There was no significant correlation between the total distance run and the number of DCX-positive cells, $R^2 = .02$.

Exercise Facilitates Y-Maze Acquisition, Retention, and Reversal Learning

We investigated the consequences of 14 days of voluntary wheel running on various aspects of Y-maze learning. First, the effects of wheel running on acquisition of the Y maze were studied (see Figure 2A). The distance run by mice that were trained in the Y maze and by runners that were not trained was similar (153 ± 10 and 156 ± 7 km, respectively). Overall, runners performed significantly better than sedentary mice (see Figure 2B), repeated measures ANOVA, $F(1, 14) = 30.1$, $p \leq .001$; Session \times Group interaction, $F(7, 98) = 3.88$, $p \leq .001$. A more detailed examination of the first session (see Figure 2C) shows that runners did not have an initial bias for the correct arm but readily learned the position of the food reward within the first training session, repeated measures ANOVA, $F(1, 14) = 13.5$, $p \leq .01$.

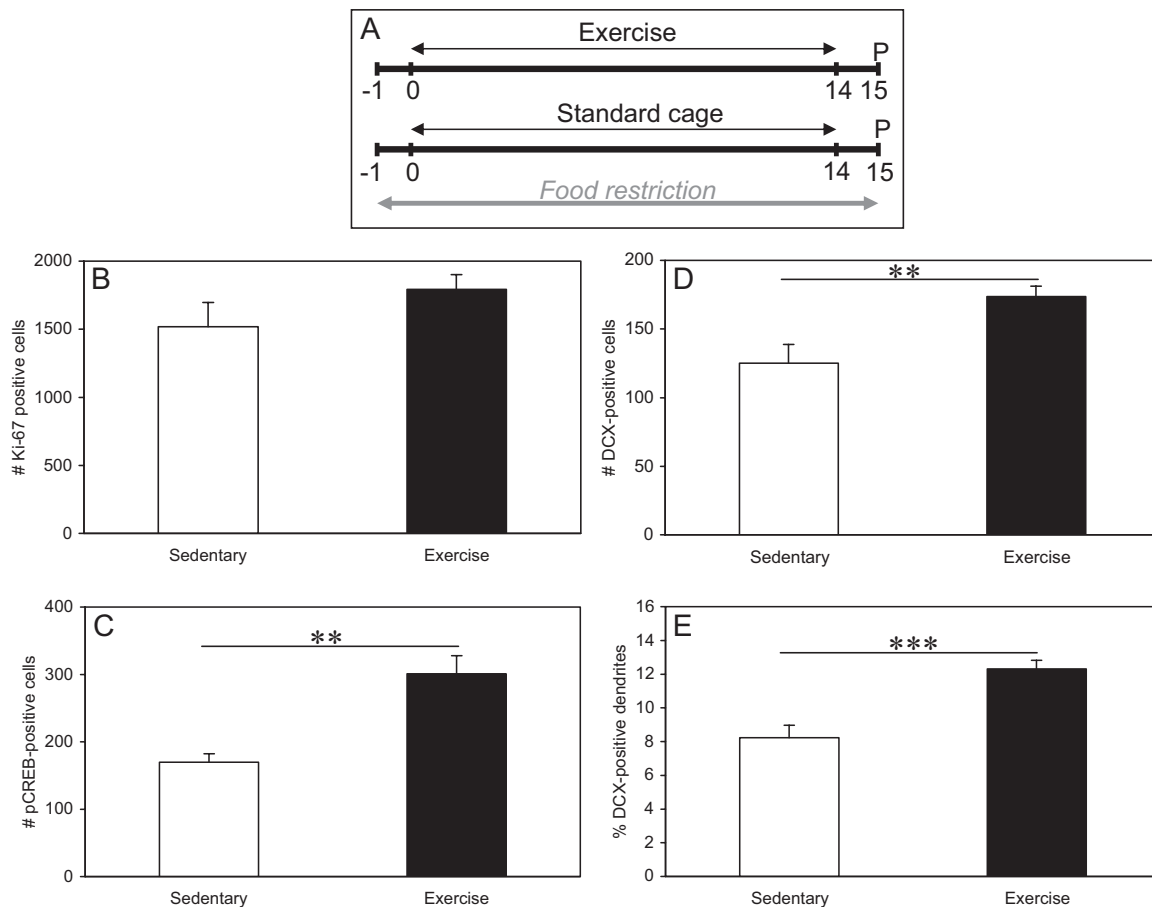


Figure 1. Effect of exercise on hippocampal neurogenesis and CREB phosphorylation (sedentary, $n = 7$; runners, $n = 8$). A: Overview of the experimental protocol. Before the runners were housed with a running wheel, they were kept in standard cages. P = perfusion; numbers indicate days. B: No difference was found in the number of proliferating cells in the subgranular zone, measured by Ki-67-positive cell numbers. C: After 14 days of running-wheel access, the exercise group's granule cell layer of the hippocampus contained significantly more Ser133-phosphorylated CREB (pCREB)-positive cells than did that of the sedentary mice. D: The 14-day exercise procedure resulted in a significant increase in the number of doublecortin (DCX)-positive cells and DCX-positive dendrites. E: Density of DCX-positive dendrites. Error bars represent SEM. ** $p < .01$. *** $p < .001$.

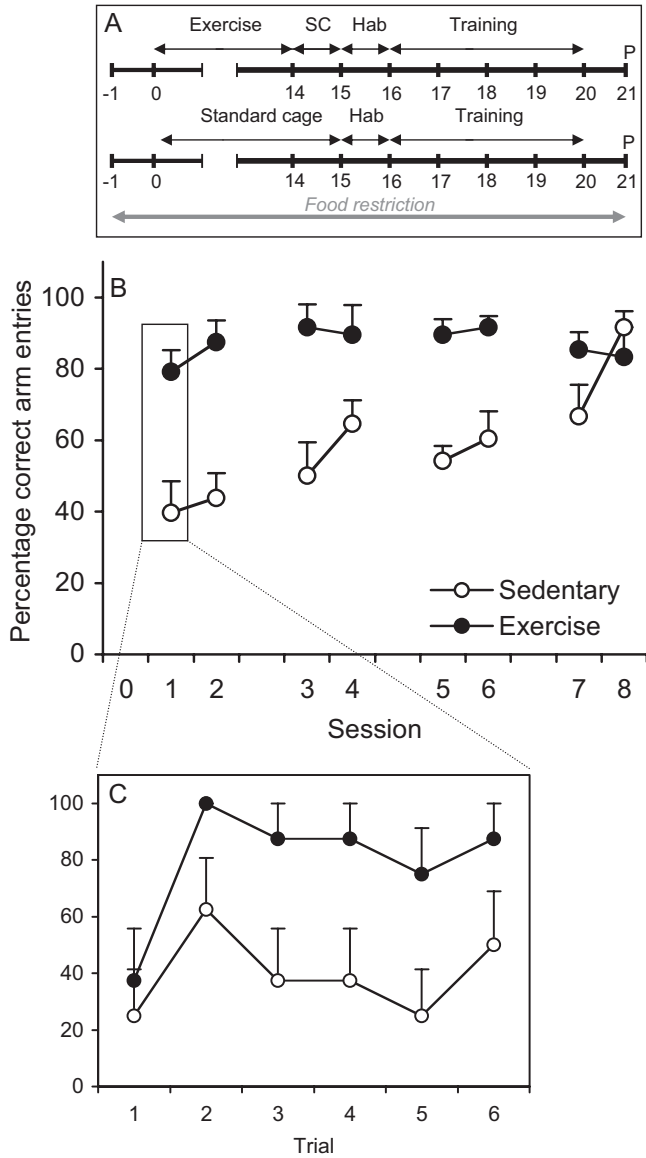


Figure 2. Effect of exercise on Y-maze acquisition (sedentary, $n = 8$; runners, $n = 8$). A: Schedule of the experimental protocol that was used. The upper line shows the experimental procedure for the runners; the lower one represents the sedentary mice. Before the runners were housed with a running wheel, they were kept in standard cages. SC = standard cage; Hab = habituation to the Y maze; P = perfusion; numbers indicate days. B: The learning curves show that mice with enhanced physical activity (runners) performed significantly better in the Y maze than sedentary mice. The learning curves show the average percentage of correct arm choices per session of six trials \pm SEM. C: Because the runners performed above chance level during the first session, this session was subdivided into the individual trials. The graph shows that runners did not have an initial preference for the baited arm but that they quickly learned which arm contained the food. The learning curves show the average percentage of mice that entered the correct arm per trial \pm SEM.

Next, we investigated whether 14 days of exercise, starting directly after Y-maze training was completed, had an effect on memory retention (see Figure 3A). Mice that had access to a wheel ran in total 110 ± 10 km. Mice quickly relearned the position of

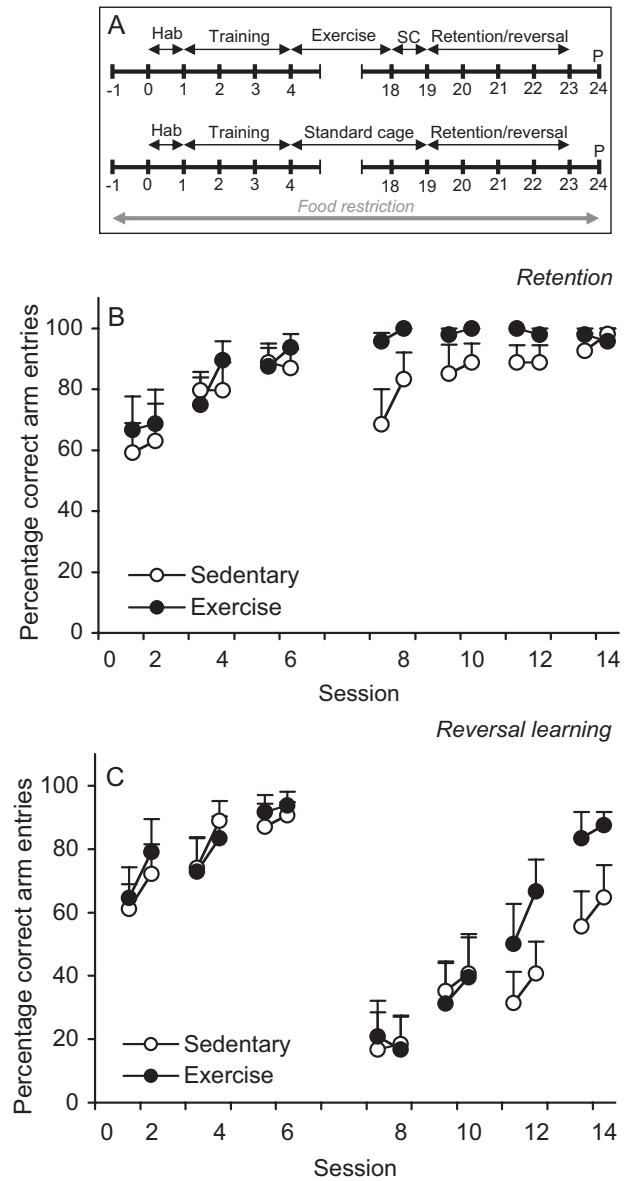


Figure 3. Effect of exercise on memory retention and reversal learning. A: Schematic overview of the experimental procedure. The upper line represents the experimental protocol for runners; the lower line shows the procedure for sedentary mice. Before the runners were housed with a running wheel, they were kept in standard cages. Hab = habituation to the Y maze; SC = standard cage; P = perfusion; numbers indicate days. B: All mice were fully trained in the Y maze. Note that none of the mice had access to a running wheel at the moment of training. Both groups were treated similarly during training. Directly after the last training session (Session 6), mice were either housed with a running wheel ($n = 8$) or kept in a standard cage ($n = 9$) for 14 days. After this 14-day interval, mice with enhanced physical activity performed significantly better in the retention test (starting at Session 7) compared with sedentary controls. C: Also in the reversal task, in which the food was located in the arm opposite to the one during training, runners ($n = 8$) showed an improved performance compared with sedentary mice ($n = 9$). The learning curves show the average percentage of correct arm choices per session of six trials \pm SEM.

the food reward (repeated measures ANOVA, session effect, $p \leq .05$). Furthermore, there was a significant Session \times Group interaction, $F(7, 105) = 2.4$, $p \leq .05$, indicating that running-wheel activity facilitated Y-maze retention (see Figure 3B).

Third, the effects of running-wheel activity on reversal learning were tested. A similar protocol was used as for the retention test (see Figure 3A), except for the fact that the food was placed in the arm that was opposite to the one that was rewarded during training. The runners ran a distance of 104 ± 10 km. In the first reversal session, both sedentary and exercise mice showed a reduced performance, which was significantly improved during the following sessions (see Figure 3C; $p \leq .001$). The significant Session \times Group interaction, $F(7, 105) = 2.1$, $p \leq .05$, showed that runners learned to find the new location of the food faster than sedentary mice.

The reversal effect (i.e., the performance in the first reversal session) did not differ between runners and sedentary mice. On the basis of the perfect retention score of the runners and the impaired performance of sedentary mice in the first few retention sessions, a stronger reversal effect in runners and a weaker effect in seden-

tary mice might have been expected. Therefore, we tested with an independent samples t test to determine whether there was a discrepancy between the performance in the first retention session and the performance in the first reversal session. In order to be able to compare performance in these two paradigms, we subtracted the percentage of correct arm entries during reversal learning from 100% and compared these values with the retention score. There was no significant difference between the first retention session and the reversal effect for either the runners ($p = .56$) or the sedentary mice ($p = .17$), indicating that the memory for the correct arm did not differ between the retention and the reversal groups.

DCX and pCREB Are Colocalized in the Subgranular Zone

Brains were stained for the immature neuron marker DCX and for pCREB (see Figure 4). DCX is a suitable marker for investigating the absolute number of newly formed neurons (Rao & Shetty, 2004). CREB phosphorylation has also been reported to

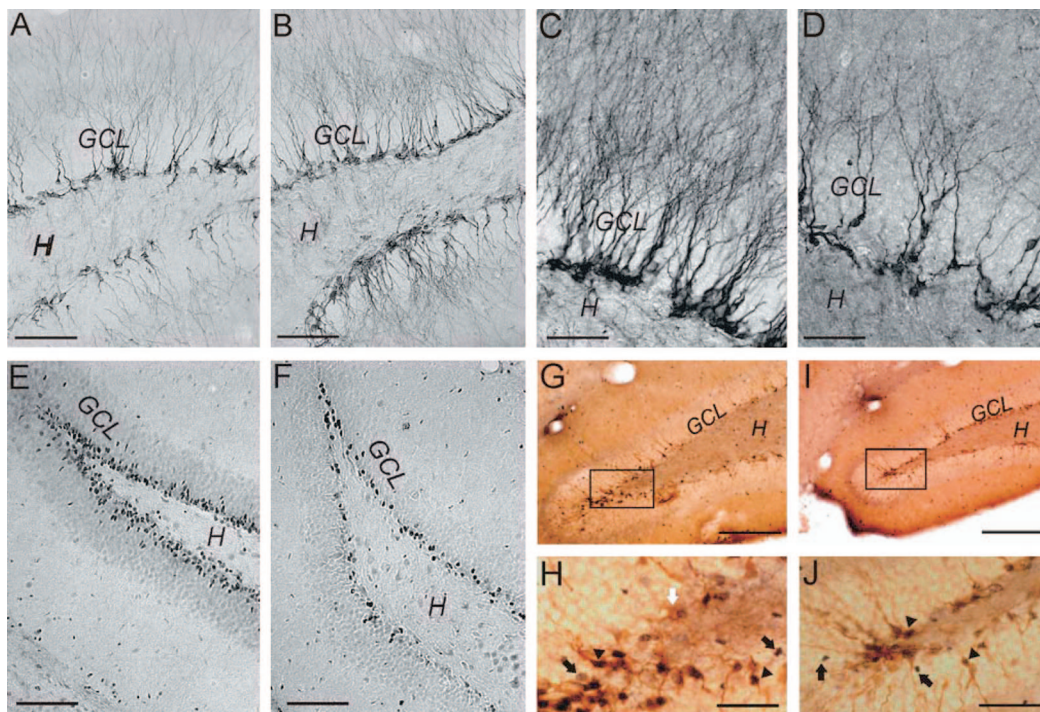


Figure 4. Ser133-phosphorylated CREB (pCREB) and doublecortin (DCX) immunoreactivity. DCX expression is significantly increased after 14 days of exercise (B), compared with sedentary controls (A). C: Close-up of DCX-immunoreactive neurons in the inner blade of a runner after training in the Y maze. D: Comparable picture as in C, but now for a mouse that has been trained, subsequently housed with a running wheel, and finally trained in the reversal learning task. E: pCREB-immunoreactive cells in runner that was naive for the Y maze. F: pCREB-positive cells in a mouse that has been trained, subsequently housed with a running wheel, and finally tested in the retention task. G, I: A strong colocalization was observed between pCREB (dark blue) and DCX (brown). Staining was performed in experimentally naive, sedentary mice. H, J: Magnification of the selected area in G and I, respectively. Black arrowheads point toward examples of DCX and pCREB-double positive cells, which can be distinguished from DCX-single labeled cells (white arrow) by their dark brown nuclear staining. Black arrows indicate pCREB-positive, but DCX-negative cells. H = hilus; GCL = granule cell layer. Scale bar: 100 μm for G and I; 50 μm for A, B, E, F, H, and J; 25 μm for C and D.

occur in newly formed, PSA-NCAM (neural cell adhesion molecule) positive granule neurons during maturation (Nakagawa et al., 2002; Zhu, Lau, Liu, Wei, & Lu, 2004). Because there is an almost complete overlap between DCX expression and NCAM polysialylation (Nacher, Crespo, & McEwen, 2001), we supposed that the pCREB-positive cells in the granule cell layer, located at the border with the hilus, were in fact newly formed neurons. This assumption was confirmed by the significant correlation that we found between the number of DCX-expressing neurons and the number of pCREB-positive cells, $R^2 = .62$, $p \leq .001$. In addition, double labeling for DCX and pCREB showed that the majority of the DCX-positive cells colocalized with pCREB (see Figure 4). Occasionally, a DCX single labeled cell could be found. However, not all pCREB positive cells coexpressed DCX, suggesting that some existing mature granule neurons contain pCREB.

Y-Maze Retention and Reversal Learning Reduce Hippocampal Neurogenesis

Because we observed that running-wheel activity enhanced neurogenesis and facilitated acquisition and retrieval of spatial information, we further explored the relationship between exercise, hippocampal neurogenesis, and Y-maze training. In order to statistically analyze potential effects of exercise or any of the Y-maze conditions on the number of newly formed hippocampal neurons, we performed a two-way ANOVA with the four different Y-maze groups (naive, acquisition, retention, and reversal) and the housing condition (sedentary or exercise) as between-subjects variables. Analysis of the Ki-67 staining (see Figure 5A) revealed a significant effect of exercise, $F(1, 61) = 4.9$, $p \leq .05$, with sedentary mice having more Ki-67-positive cells ($2,083 \pm 104$) than did runners ($1,797 \pm 86$). The different Y-maze paradigms did not affect the number of proliferating cells in the hippocampus, $F(3, 61) = 0.6$, $p = .65$. Furthermore, there was no interaction between exercise and Y-maze training, $F(3, 61) = 2.2$, $p = .10$.

For pCREB (see Figure 5B), $F(1, 61) = 40.6$, $p \leq .001$; DCX-positive cell number (see Figure 5C), $F(1, 61) = 36.0$, $p \leq .001$; and DCX-positive dendrite density (see Figure 5D), $F(1, 61) = 36.4$, $p \leq .001$, a significant effect of exercise was observed, with runners having more DCX and pCREB immunoreactivity. The average number of pCREB-positive cells in runners was 164 ± 12 , versus 88 ± 8 in sedentary mice. Runners had on average 142 ± 7 DCX-positive cells per hippocampal section and a DCX-positive dendrite density of $11 \pm 0.6\%$ against 102 ± 6 DCX-positive cells and $8 \pm 0.5\%$ DCX-positive dendrites in sedentary mice, respectively.

The Y-maze groups also differed significantly from each other with respect to pCREB-positive cell number, $F(3, 61) = 11.2$, $p \leq .001$; DCX-positive cell number, $F(3, 61) = 20.2$, $p \leq .001$; and DCX-positive dendrite density (see Figure 5D), $F(3, 61) = 36.4$, $p \leq .001$. Furthermore, regression analysis showed that the DCX-positive dendrite measurements provide a reliable measure for the number of DCX-positive cells, $R = .75$, $p \leq .001$. There was no significant interaction between exercise and the different Y-maze paradigms for any of the three stainings: pCREB, $F(3, 61) = 2.0$, $p = .13$; DCX-positive cells, $F(3, 61) = 0.8$, $p = .51$; and DCX-positive dendrites, $F(3, 61) = 1.8$, $p = .15$. A post hoc Tukey's honestly significant difference test was performed to test which of the Y-maze groups differed from each other.

The memory retention test significantly reduced the number of pCREB-positive cells (88 ± 11 cells), DCX-positive cells (96 ± 8 cells), and DCX-positive dendrite density ($7 \pm 0.6\%$) compared with naive controls (pCREB: 168 ± 17 cells, DCX: 159 ± 7 cells and $12 \pm 0.5\%$ dendrites, $p \leq .001$ for the three analyses) and memory acquisition (pCREB: 156 ± 22 cells, DCX: 137 ± 13 cells and $11 \pm 1\%$ dendrites, $p \leq .001$ for all three analyses). Also, reversal learning caused a significant decrease in the number of pCREB-positive cells (95 ± 10 cells), DCX-positive cells (99 ± 7 cells), and DCX-positive dendrite density ($7 \pm 0.5\%$) relative to naive controls ($p \leq .001$ for all three analyses) and mice that were trained in the memory acquisition test ($p \leq .001$ for DCX-positive dendrite density, $p = .002$ for pCREB and DCX-positive cell number).

Discussion

Beneficial Effects of Exercise on Y-Maze Acquisition, Retention, and Reversal Learning

Our data show that 14 days of exercise increased the rate of acquisition in the Y maze, improved retention of previously acquired information, and facilitated reversal learning. The fact that exercise had a positive effect on Y-maze acquisition is in line with other studies, using different learning tasks (Fordyce & Farrar, 1991; Fordyce & Wehner, 1993; Van Praag, Christie, et al., 1999). We show for the first time that physical exercise not only promotes the acquisition of a spatial learning task but also is beneficial for the retrieval of spatial reference memory.

Previous studies have also reported beneficial effects of running on memory retention (Burghardt et al., 2006; Samorajski et al., 1985). However, in these studies, mice had access to a running wheel prior to acquisition of the task. This means that, in these cases, the improved memory retention could be due to the exercise-induced enhancement of memory formation or an improvement of memory retrieval. In the present study, we made a distinction between the effects of exercise on memory formation and memory retrieval by providing the running wheel either before or after acquisition of the Y-maze task. In this way, we could show that an increase in physical activity can promote memory retention by facilitating memory retrieval.

Wheel running also facilitated reversal learning. During reversal learning, an animal has to adapt previously acquired information to a new situation. Because many of the features of the task that were acquired during training are still valid, the old memory trace does not need to be erased from the brain. Instead, the animal needs to modify the existing memory trace, which requires correct retrieval of the memory and the flexibility to unlearn certain details of the task, or to inhibit the acquired response and to relearn new facts. Here we show that mice that had been exposed to a running wheel for 14 days prior to the reversal learning task were significantly faster in acquiring the new position of the food in the Y maze than sedentary mice. These data suggest that enhanced physical activity has a positive effect on the behavioral flexibility of a mouse. The performance in the memory acquisition task, memory retention or reversal learning did not correlate with the total distance run by the mice.

There are a variety of potential mechanisms that can cause the exercise-induced improvements in learning and memory. The ben-

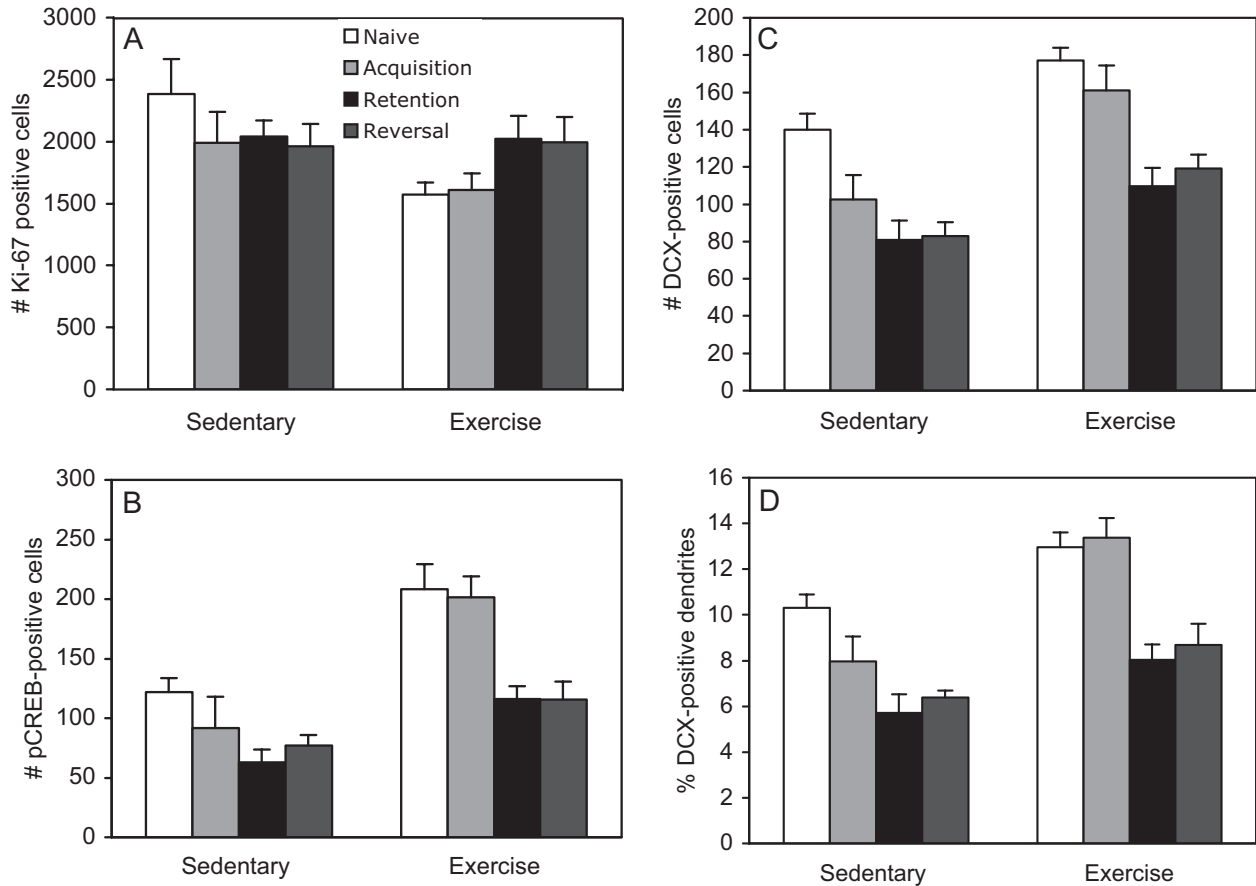


Figure 5. Effect of acquisition on hippocampal neurogenesis (naive sedentary mice, $n = 8$; naive runners, $n = 8$; sedentary acquisition, $n = 7$; runner acquisition, $n = 7$; sedentary retention, $n = 8$; runner retention, $n = 8$; sedentary reversal, $n = 9$; runner reversal, $n = 7$). A: The number of Ki-67-positive cells was significantly decreased in runners, compared with sedentary mice. None of the Y-maze paradigms caused any change in the number of hippocampal proliferating cells. B: Runners had significantly more Ser-133-phosphorylated CREB (pCREB)-positive cells than sedentary controls. Acquisition of the Y maze did not cause changes in the number of pCREB-positive cells, but memory retention and reversal learning caused a significant reduction compared with naive controls and memory acquisition. C: Doublecortin (DCX)-positive cell number was significantly higher in runners compared with sedentary mice. Y-maze acquisition did not influence DCX-positive cell number, but the retention test and the reversal learning task significantly reduced DCX-positive cell number compared with naive controls and memory acquisition. D: The density of DCX-positive dendrites in the granule cell layer was significantly enhanced after exercise but remained unchanged after memory acquisition. A significant reduction was observed after memory retention and reversal learning, when compared with naive mice or the acquisition group. Statistical details can be found in the text. For Ki-67, cell numbers are shown as the total number of immunopositive cells/dentate gyrus \pm SEM. For the other pCREB and DCX, cell numbers are shown as the average number of cells/section \pm SEM.

official effects of wheel running on Y-maze performance in runners could be due to increased transcription or phosphorylation of genes that promote long-term memory formation, such as CREB (Bourtchuladze et al., 1994; Guzowski & McGaugh, 1997; Kida et al., 2002). We have shown an increase in pCREB-positive cell number after 14 days of exercise, which exceeded the increase in the number of DCX-positive cells, indicating that running also induced CREB phosphorylation in other cells than the newly formed granule neurons. In addition, running has been shown to elevate levels of various growth factors and neurotrophic factors (Fabel et al., 2003; Farmer et al., 2004; Gomez-Pinilla, Dao, & So,

1997; Oliff, Berchtold, Isackson, & Cotman, 1998; Trejo, Carro, & Torres-Aleman, 2001) and to promote, for instance, cerebral blood flow (Endres et al., 2003), angiogenesis (Swain et al., 2003) and cholinergic synaptic communication (Fordyce & Farrar, 1991), all of which may positively influence learning and memory. As the control mice did not have a locked running wheel in the cage, the effects of exercise could also partially be caused by the enrichment of having a wheel in the cage and not by the enhanced physical activity itself (Pietropaolo, Feldon, Alleva, Cirulli, & Yee, 2006). It could therefore be worthwhile for future studies to use a locked wheel as a control.

Exercise Promotes Hippocampal Neurogenesis

Another phenomenon that might underlie the mnemonic effects of wheel running is the exercise-induced increase in hippocampal neurogenesis. We show here that 14 days of exercise, in combination with dietary restriction, increased the number of maturing granule neurons in the hippocampus. Newborn neurons form a specific population of cells that may serve as a substrate for the formation of new memories. They are not yet involved in other memory traces, and they show extraordinary morphological (Hastings & Gould, 1999; Seki & Arai, 1991, 1993) and synaptic (Schmidt-Hieber, Jonas, & Bischofberger, 2004; Snyder, Kee, & Wojtowicz, 2001) plasticity. They could therefore play a specific role in the formation of memories.

It has previously been shown that the positive effects of environmental enrichment on memory formation are dependent on the increase in neurogenesis that occurs under these housing conditions (Bruehl-Jungerman, Laroche, & Rampon, 2005). This suggests that the same principle might be true for the exercise-mediated enhancement of learning. However, when studying mice instead of rats and applying a different learning task, others showed that the beneficial effects of an enriched environment on cognitive processes do not require an increase in neurogenesis (Meshi et al., 2006). In addition, a study using mice that were selectively bred for high levels of wheel running showed an exercise-induced increase in neurogenesis that reached a plateau level but no corresponding improvement in Morris water maze learning (Rhodes et al., 2003). Thus, additional research will be necessary to determine whether the exercise-induced enhancement of neurogenesis contributes to the positive effects of exercise on Y-maze performance.

Our data on exercise and memory retention seem to contradict those of Feng et al. (2001), who stated that hippocampal neurogenesis might serve to remove outdated information from the hippocampus. Feng and coworkers reported that an increase in neurogenesis after acquisition of a learning task impairs memory retention, whereas we see an improvement. However, there are major differences in the experimental set-up between the present study and the experiments by Feng and colleagues. We used another learning task (Y maze versus contextual fear conditioning), a different method to increase neurogenesis (wheel running versus environmental enrichment), and wildtype mice instead of knockout mice. Nevertheless, with our experimental paradigm we cannot confirm that hippocampal neurogenesis erases existing memory traces from the hippocampus.

It cannot be excluded that the beneficial effects of exercise on learning are influenced by an interaction between running and the caloric restriction protocol that we used. Food restriction itself has been reported to have beneficial (Magnusson, 2001) or disadvantageous (Yanai, Okaichi, & Okaichi, 2004) effects on learning and memory, and it stimulates neurogenesis (Lee, Duan, Long, Ingram, & Mattson, 2000). Sedentary mice were also subjected to dietary restriction, so we do not expect the food restriction per se to play a major role in the exercise-induced improvement in Y-maze performance. However, it is possible that food restriction enhanced or perturbed the effects of exercise on neurogenesis and learning (Mattson, 2000).

This might also be one of the reasons why we did not observe an increase in cell proliferation in our combined exercise and food

restriction protocol. Perhaps, under conditions of restricted caloric intake, neurogenesis is regulated in a different way than under ad-lib conditions. Another explanation for the lack of a proliferation effect, or even a reduction in proliferation in some of the mice after exercise, could be that our mice were individually housed. A recent study has shown that social isolation delays the effects of exercise on hippocampal cell proliferation (Stranahan, Khalil, & Gould, 2006). However, we have shown previously that exercise in individually housed mice, with ad-lib food availability, increases cell proliferation after 9 days of running (Van der Borght et al., 2006).

Memory Retention and Reversal Learning Reduce Hippocampal Neurogenesis in Both Sedentary Mice and Runners

We further determined the effects of training, memory retention, and reversal learning in the Y maze on hippocampal neurogenesis. The Y-maze task as used in the present study, with one baited arm throughout the experiment, is a so-called "reference memory task." This type of test is considered to be hippocampus dependent. Various reports have shown that bilateral cytotoxic lesions of the hippocampal formation result in severe impairments in the Y-maze reference memory task (Bannerman, Deacon, Seeburg, & Rawlins, 2003; Deacon, Bannerman, Kirby, Croucher, & Rawlins, 2002; Reisel et al., 2002).

We show that training in the Y maze did not cause changes in the number of immature neurons. These data confirm the results of other studies, partly from our group, in which spatial learning had no effect on hippocampal neurogenesis (Snyder et al., 2005; Van der Borght et al., 2005; Van Praag, Kempermann, et al., 1999). We further explored the effects of memory retention and reversal learning on hippocampal neurogenesis. Both paradigms caused a dramatic reduction in the number of maturing neurons, both in sedentary control mice and in runners. The proliferation rate of hippocampal progenitors, which was analyzed by counting Ki-67-positive nuclei, was not influenced by any of the Y-maze paradigms. The most probable explanation for the decrease in the number of newly formed neurons is increased apoptosis of newly formed cells. Alternatively, the generation of new cells may have been inhibited during retention testing and reversal learning. This option may be less likely, because the Ki-67 staining did not show any differences in the generation of new cells between the experimental groups. A third option is that memory retrieval caused an accelerated maturation of neural precursors, which would reduce the time window of approximately 6 days during which DCX is normally expressed (Kempermann, Jessberger, Steiner, & Kronenberg, 2004). However, because the length of DCX expression has been shown to remain stable, even in conditions such as aging or hippocampal injury (Rao & Shetty, 2004), the latter explanation is not very probable.

The dramatic changes we observed in the number of immature neurons were similar for mice exposed to the retention or the reversal task, suggesting that there is a common factor in these two paradigms that causes these changes. One of the common features of memory retention and reversal learning is that both require retrieval of previously acquired information. Other studies have shown a reduction in hippocampal activity after memory retrieval. Retention testing of mice in a radial maze, 25 days after acquisi-

tion, decreased hippocampal metabolic activity, measured by (^{14}C)-deoxyglucose uptake, below baseline levels (Bontempi, Laurent-Demir, Destrade, & Jaffard, 1999). In addition, repeated exposure of rats to a familiar environment significantly reduced hippocampal CREB phosphorylation compared with naive controls (Winograd & Viola, 2004). The reduced neurogenesis after memory retrieval could also be an indication of suppressed hippocampal activity.

A potential explanation for the reduced neurogenesis is that it might help to prevent interference between existing memory traces and new experiences. Fourteen days after the mice have mastered the Y-maze task, the acquired information is most likely stored in the cortex and retrieval is independent of the hippocampus (Beylin et al., 2001; Kim & Fanselow, 1992; Takehara, Kawahara, & Kirino, 2003). However, reexposure to the same context is thought to reactivate the hippocampal memory trace and to return it to a more labile state, which is sensitive to disruption (Debiec, Ledoux, & Nader, 2002; Nader, 2003). It might be important for the optimal retrieval of memories to partly inhibit neurogenesis when memories are in this labile state. Because newborn neurons may form an important substrate for the formation of new memory traces, we hypothesize that active suppression of neurogenesis during reexposure to a familiar environment may prevent the formation of redundant memories and reduce possible interference between the existing and the newly formed memory trace.

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