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Exercise Increases Hippocampal Neurogenesis to High Levels but Does Not Improve Spatial Learning in Mice Bred for Increased Voluntary Wheel Running

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The hippocampus is important for the acquisition of new memories. It is also one of the few regions in the adult mammalian brain that can generate new nerve cells. The authors tested the hypothesis that voluntary exercise increases neurogenesis and enhances spatial learning in mice selectively bred for high levels of wheel running (S mice). Female S mice and outbred control (C) mice were housed with and without running wheels for 40 days. 5-Bromodeoxyuridine was used to label dividing cells. The Morris water maze was used to measure spatial learning. C runners showed a strong positive correlation between running distance and new cell number, as well as improved learning. In S runners, neurogenesis increased to high levels that reached a plateau, but no improvement in learning occurred. This is the first evidence that neurogenesis can occur without learning enhancement. The authors propose an alternative function of neurogenesis in the control of motor behavior.

The hippocampus plays an important integrative role in the central nervous system. It receives information from each of the sensory modalities and projects widely throughout the brain (Swanson, 1983). It is most well known for its role in learning and memory (Biegler, McGregor, Krebs, & Healy, 2001; Deweer, Pillon, Pochon, & Dubois, 2001; Fortin, Agster, & Eichenbaum, 2002; Suzuki & Clayton, 2000; Wittenberg & Tsien, 2002). However, a minority of studies have also identified roles for the hippocampus in motivation (Tracy, Jarrard, & Davidson, 2001) and motor behavior (Morris & Hagan, 1983; Oddie & Bland, 1998; Vanderwolf, 1969).

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The adult mammalian dentate gyrus (DG) of the hippocampus generates neural progenitors that can differentiate into new neurons (Gage, 2000). This feature of being able to grow new neurons (Gage, 2000) is unusual, because most neurons are terminally differentiated and, when they die, are not replaced (Gage, Kempermann, Palmer, Peterson, & Ray, 1998). In addition to DG, neurogenesis occurs in the olfactory lobe (Gage et al., 1998). It has also been suggested that neurogenesis occurs in the neocortex (Gould, Reeves, Graziano, & Gross, 1999), although the validity of this claim has recently been challenged on methodological grounds (Rakic, 2002). In contrast, neurogenesis in DG is well established (van Praag et al., 2002).

The process of neurogenesis in DG, which includes proliferation, survival, migration, and differentiation (van Praag et al., 2002), is influenced by a variety of factors and experiences, such as environmental enrichment (Kempermann, Kuhn, & Gage, 1997b), exercise (Trejo, Carro, & Torres-Aleman, 2001; van Praag, Christie, Sejnowski, & Gage, 1999; van Praag, Kempermann, & Gage, 1999) and stress (Gould, 1994; Lemaire, Koehl, Le Moal, & Abrous, 2000). In addition, brain injuries caused by lesions (Zuo, 1998), stroke (Arvidsson, Kokaia, & Lindvall, 2001), ischemia (Jin et al., 2001), or epilepsy (Parent et al., 1997) affect numbers of new cells. Depression (Jacobs, van Praag, & Gage, 2000) and psychoactive drugs (Malberg, Eisch, Nestler, & Duman, 2000) have also been suggested to influence neurogenesis. On the basis of these studies, roles for the new cells in cognition, brain repair, and mental health have been proposed (Gage et al., 1998; Gould, Beylin, Tanapat, Reeves, & Shors, 1999; Jacobs et al., 2000).

Exercise increases cell proliferation and survival in the DG of adult mice (Trejo et al., 2001; van Praag, Christie, et al., 1999; van Praag, Kempermann, & Gage, 1999). Exercise-induced neuronal activity in DG (Oladehin & Waters, 2001) and hippocampal theta rhythms that control the speed of running (Oddie & Bland, 1998) may increase levels of brain-derived neurotrophic factor (BDNF) in the hippocampus (Nanda & Mack, 2000; Patterson, Grover, Schwartzkroin, & Bothwell, 1992; Zafra, Hengerer, Leibrock, Thoenen, & Lindholm, 1990) and stimulate hippocampal neurogenesis (Lee, Duan, Long, Ingram, & Mattson, 2000; Pencea, Bingaman, Wiegand, & Luskin, 2001). This concept is consistent with a strong positive correlation between running distance, hippocampal BDNF mRNA (Neeper, Gomez-Pinilla, Choi, & Cotman, 1995), and the number of new cells produced (Allen et al., 2001), and suggests a potential causal link between exercise, BDNF, and neurogenesis in the hippocampus.

In addition to increasing neurogenesis, exercise improves spatial memory in rodents (Anderson et al., 2000; Fordyce & Wehner, 1993; van Praag, Christie, et al., 1999), and it has been suggested that exercise-induced hippocampal neurogenesis contributes to the learning enhancement (van Praag, Christie, et al., 1999). However, physical activity is associated with a variety of changes that may enhance synaptic plasticity independently from neurogenesis, such as increases in acetylcholine (Fordyce & Farrar, 1991), opiate (Sforzo, Seeger, Pert, Pert, & Dotson, 1986), and monoamine (Meeusen et al., 1997) neurotransmitters; the transcription factor *c-fos* (Oladehin & Waters, 2001); insulin-like growth factor (Carro, Nunez, Busiguina, & Torres-Aleman, 2000), fibroblast growth factor (Gomez-Pinilla, So, & Kesslak, 1998), and BDNF (Neeper et al., 1995; Widenfalk, Olson, & Thoren, 1999).

Recently, through a selective breeding strategy, we generated mice that display increased voluntary wheel-running behavior (S mice). The mice in our model are highly motivated for exercise on running wheels (Garland, 2003; Girard, McAleer, Rhodes, & Garland, 2001). We used these mice to test the hypothesis that the high levels of exercise increase hippocampal BDNF protein concentration and neurogenesis, and enhance learning in the Morris water maze. This study is unique because the S mice run up to 18 km/day, which is approximately 2-fold higher than that observed in wild house mice (Dohm, Richardson, & Garland, 1994) and approximately 2.7-fold higher than in typical laboratory mice such as the random-bred control lines (C mice) of this study (Garland, 2003). Thus, our model allows a unique assessment of the affects of high levels of voluntary physical activity on markers of neuronal plasticity and learning.

Method

Subjects

We studied female mice from Generations 25 and 27 of an artificial selection experiment for high voluntary wheel-running behavior (Swallow, Carter, & Garland, 1998). The original progenitors were outbred, genetically variable laboratory house mice (*Mus domesticus*) of the Hsd:ICR strain. After two generations of random mating, mice were randomly paired and assigned to eight closed lines (10 pairs in each). In each subsequent generation, when the offspring of these pairs were 6–8 weeks old, they were housed individually with access to a running wheel for 6 days. Daily wheel-running activity was monitored by an automated system.

In the four S lines, the male and female with the highest running levels from each family were selected as breeders to propagate the lines to the next generation. Wheel running was quantified as the total number of revolutions run on Days 5 and 6 of the 6-day test. In the four C lines, a male and a female were randomly chosen from each family. Within all lines, the chosen breeders were randomly paired, except that sibling matings were not allowed.

To supply mice for the experiments presented here, Generation 24 and 26 parents were allowed to produce a second litter (i.e., Generations 25 and 27). As resources were limited, only females in these second litters were used. The mice were housed individually either with or without access to a running wheel (1.12 m circumference) for 40 days, starting from a mean ($\pm$ *SD*) age of 29 $\pm$ 1.9 days. During the first 10 days, mice were given intraperitoneal injections of 5-bromodeoxyuridine (BrdU, 50 mg/kg, 1 injection per mouse per day). During the last 6 days, mice were trained in the Morris water maze (see *Spatial Learning* below) and then transcardially perfused or killed by isoflurane overdose. Generation 25 mice (C nonrunners, $n = 24$; C runners, $n = 24$; S nonrunners, $n = 24$; S runners, $n = 24$) were processed for hippocampal neurogenesis and volume of DG. Generation 27 mice (C nonrunners, $n = 18$; C runners, $n = 18$; S nonrunners, $n = 24$; S runners, $n = 24$) were processed for hippocampal BDNF concentration and Morris water maze learning.

The guidelines published in *Principles of Laboratory Animal Care* (NIH publication No. 85-23, revised 1985) were followed, and all experiments were approved by the University of Wisconsin Animal Care Committee. Throughout the selection experiment and during this study, water and food were available ad libitum. Rooms were controlled for temperature (~22° C) and photoperiod (12-hr light–dark cycle).

Immunohistochemistry

After the 40-day experimental trial, all Generation 25 mice were deeply anesthetized with sodium pentobarbital and then perfused transcardially with 4% (wt/vol) paraformaldehyde. Brains were removed, postfixed in 4% paraformaldehyde for 24 hr, and placed in 30% (wt/vol) sucrose. A sliding microtome was used to cut coronal sections (40 μm), which were placed in consecutive order into a 96-well plate containing tissue cryoprotectant solution (25% ethylene glycol, 25% glycerin, and 0.05 M phosphate buffer), then stored at −20° C. Immunohistochemistry for BrdU, the neuronal marker NeuN (Mullen, Buck, & Smith, 1992), and the glial marker calcium binding protein S100β (Boyes, Kim, Lee, & Sung, 1986) was performed on free-floating sections that were pretreated by denaturing DNA, as described previously (Kuhn, Dickinson-Anson, & Gage, 1996). The antibodies were mouse anti-BrdU (1:400; Boehringer Mannheim, Indianapolis, IN), and for immunofluorescent triple labeling, rat anti-BrdU ascites fluid (1:100; Accurate, Harlan Sera-Lab, Loughborough, England), rabbit anti-S100β (1:2,500; Swant, Bellinoza, Switzerland), and mouse anti-NeuN (1:20; kindly provided by R. J. Mullen, University of Utah). To determine the number of BrdU-labeled cells, we stained for BrdU with the peroxidase method (ABC system with biotinylated donkey anti-mouse IgG antibodies and diaminobenzidine as chromogen; Vector Laboratories, Burlingame, CA). The fluorescent secondary antibodies used were FITC-labeled anti-mouse IgG, Cy3-labeled anti-rat IgG, and Cy5-labeled anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA), 6 μl/ml each.

Cell Counting and DG Volume Estimation

All counting and volume estimation were done without awareness of experimental conditions. A series of sections with a 240-μm space between sections (a 1-in-6 series), covering the entire rostrocaudal extent of DG were stained for BrdU by the peroxidase method (described above). BrdU-positive cells were counted in these sections through a 20× objective (Leitz) at a final magnification of 200×. To measure granule cell layer volume and quantitate the total number of granule cell neurons, we stained an additional 1-in-6 series of adjacent sections with 0.5 mg/ml Hoechst 33342 in Tris-buffered saline (Molecular Probes, Eugene, OR). We used a semiautomated stereology system (Stereoinvestigator; MicroBrightField,

Williston, VT) to estimate DG volume and total number of granule neurons. Sections were viewed through a 40× objective (Leitz, final magnification 400x), projected onto a computer monitor. A two-dimensional area of the DG, on each section, was traced by using the Stereoinvestigator (MicroBrightField) software. Granule cell layer volume was calculated by multiplying the distance between the sections (240 μm) by the sum of the traced granule cell layer areas (in square microns). The estimates of granule neuron number were based on optical dissector samples made according to a "fractionator" sampling scheme (West, Slomianka, & Gundersen, 1991). A box, 20 μm × 20 μm × 15 μm, was placed randomly at approximately 15 sites per section. At each site, number of cells within the box was counted by focusing through the section and counting nuclei only as they come into sharp focus. During the counting, cells in the uppermost focal plane were disregarded. This method gives an unbiased estimate of average number of cells per cubic micron (West et al., 1991). The total number of granule cells was obtained by multiplying the average number of cells per cubic micron by the granule cell layer volume estimate.

BDNF Assay

After the 40-day experimental trial, all Generation 27 mice were killed with an overdose of Isoflurane (Abbott Laboratories, North Chicago, IL). The hippocampus was rapidly dissected and stored at −80° C. Tissue was homogenized in a cold extraction buffer containing 137 mM NaCl, 20 mM Tris (pH 8.0), 1% NP-40, 10% glycerol, 0.75 mg/ml phenylmethylsulfonylfluoride, 0.61 mg/ml sodium metavanadate, 0.1 mg/ml aprotinin, and 9.4 μg/ml leupeptin. The homogenates were acidified to pH $<$ 3 with 1N HCl, vortexed, and incubated for 15 min at room temperature, followed by neutralization to pH ~8 with 1N NaOH (Okragly & Haak-Frendscho, 1997). The homogenates were then centrifuged for 10 min at 3,500 G, and the supernatant was assayed for BDNF with commercially available sandwich ELISA (R&D Systems, Minneapolis, MN).

Morris Water Maze Learning

After 34 days of residing either with or without a wheel, half the mice from Generation 27 (C nonrunners, $n = 9$; C runners, $n = 9$; S nonrunners, $n = 12$; S runners, $n = 12$) were trained in a hidden-platform Morris water maze (122 cm diameter, 36 cm deep) with four trials per day for 6 days. The platform (10.0 cm × 10.0 cm × 0.5 cm, clear plastic PVC) was hidden 1 cm below the surface of the water and remained in the same position (in the middle of the southeastern quadrant) during all tests. The water was rendered black with the addition of 250 g of nontoxic black powder paint (Crayola brand). Water temperature was 25.60° C ± 0.62 *SD*. The room (dimensions = 357 cm × 217 cm) was dimly lit (24.8 lx) by three 60-W lights placed on the floor in opposite corners of the room. Pieces of cardboard were used to prevent reflections from the water surface that would interfere with tracking the mice. Various pieces of equipment provided contrast (visual cues) on the walls. Starting points were changed every trial. Each trial lasted a maximum of 45 s. If a mouse did not reach the platform in 45 s, it was gently guided to the platform by hand. Time to reach the platform (latency), length of the swim path, and swim speed were recorded with a video-based tracking system (San Diego Instruments).

The other half of the mice from Generation 27 (C nonrunners, $n = 9$; C runners, $n = 9$; S nonrunners, $n = 12$; S runners, $n = 12$) were trained in a visible-platform Morris water maze with two trials per day for 6 days. The visible platform was raised 1 cm above the water and displayed a flagpole (18 cm high; 5 × 10 cm flag) in the center.

Statistics

In each experiment of this study, individual mice from each of the eight lines were studied. Because the lines were separately propagated for 25+ generations, individual mice in a given generation do not represent independent data points (i.e., mice within a line are more similar to one another than mice from different lines). Therefore, the individual mice must be nested within the populations from which they arose (Henderson, 1989, 1997). To satisfy this requirement, line was always entered as a random effect, nested within the fixed effect, line type (S vs. C; for theoretical justification for this approach, see Pinheiro & Bates, 2000). Continuous variables were analyzed with the "Proc Mixed" command in SAS (SAS Institute, Cary, NC), whereas binomial variables were analyzed with the "glmmPQL" command in R (open source software, Version 1.6.2; available at http://cran.us.r-project.org/). These procedures were chosen because they use restricted maximum likelihood, which is preferred over least-squares approaches when models include random effects (Littell, Milliken, Stroup, & Wolfinger, 1996; Pinheiro & Bates, 2000).

Baseline wheel running during the 40-day experimental period was analyzed with a linear model that included the fixed factor line type, and a covariate that measured friction of the wheels (total number of revolutions produced by the wheel after being accelerated to constant velocity). Variance was estimated separately for selected and control lines. The means that are displayed for the wheel-running variables (distance, duration, and speed) were adjusted for the covariate by using the LSMEANS function in SAS (Littell et al., 1996).

The remaining data (besides wheel running) were analyzed with a two-way nested analysis of variance (ANOVA) with the fixed variables line type, wheel treatment (with or without access to a running wheel), and the interaction between wheel treatment and line type. The *F* statistics and *p* values refer to Type 1, sequential tests of the fixed variables that were entered in this order: wheel treatment, line type, then interaction. Degrees of freedom for testing the line type effect were always 1 and 6, to reflect the fact that the appropriate experimental unit for testing an effect of line type is the line ($n = 8$), not the individual mouse. Degrees of freedom in the denominator for wheel treatment and the Wheel Treatment × Line Type interaction depended on the number of individual mice. Hence, for testing the effect of wheel treatment and Wheel Treatment × Line Type interaction, the individual mouse, rather than the line, was considered to be the experimental unit (following a split plot design; Littell et al., 1996, p. 32; Pinheiro & Bates, 2000, p. 45).

Prior to analysis, BrdU cell numbers were transformed by being raised to the exponent 0.2 to stabilize the variance between wheel treatments (runner vs. nonrunner). BrdU cell numbers (untransformed) in the runners were also analyzed as a function of running distance, with separate linear relationships for C and S mice (i.e., terms in the linear model included running distance, line type, Line Type × Running Distance interaction, and line as a random effect). For phenotypic analysis of BrdU-positive cells, a logistic regression was used to generate means and standard errors for the proportions of BrdU cells differentiating into neurons, astrocytes, or neither (see Table 1). A logistic regression with line entered as a random effect was used in the R "glmmPQL" command to give the *p* values associated with comparisons of the proportions between the different treatment groups. For the water maze data, a "repeated" statement in SAS Proc Mixed (days, autoregressive covariance structure; Littell et al., 1996, p. 97) was included. Body mass was explored as a potential covariate in all analyses, but it was never significant, and so was not included in the final analyses. Stage of the estrous cycle was not measured and hence was not entered as a cofactor in analyses.

Results

Wheel Running

Twenty-seven generations of selective breeding substantially increased voluntary wheel running (see Figure 1). We studied female mice from Generations 25 and 27. During the 40-day experimental period, S mice from Generation 25 ran an average of 11.50 ± 0.49 (*SE*) km/day, whereas C mice ran 3.60 ± 0.49 km/day, $F(1, 6) = 130.7$, $p < .0001$. Both mean wheel-rotation speed (1.6 ± 0.041 km/hr vs. 0.65 ± 0.041 km/hr), $F(1, 6) =$

Table 1
Phenotypic Analysis of BrdU-Positive Cells: Mean (±SEM) Percentage of Neurons, Astrocytes, or Other From a Logistic Regression

	Nonrunner		Runner	
Phenotypes (%)	Control (n = 10)	Selected (n = 6)	Control (n = 8)	Selected (n = 7)
Neuron	88 ± 1.9	84 ± 2.7	97 ± 1.1	96 ± 1.4
Astrocyte	4 ± 1.2	7 ± 1.9	1 ± 0.6	1 ± 0.8
Other	8 ± 1.5	9 ± 2.1	2 ± 0.9	3 ± 1.1

Note. For each individual, 30 BrdU-positive cells in the dentate gyrus were analyzed for coexpression of the markers NeuN (indicating neuronal phenotype) or S100β (indicating astrocyte phenotype). BrdU = 5-bromodeoxyduridine.

245.5, $p < .0001$, and duration of wheel rotations (7.30 ± 0.31 vs. 5.20 ± 0.57), $F(1, 6) = 9.8$, $p = .02$, were greater in S mice. For Generation 27, results were similar: S mice ran 12.80 ± 1.28 km/day versus 5.70 ± 0.80 km/day in C mice, $F(1, 5) = 21.81$, $p = .006$. Although wheel friction was included as a covariate in these analyses, it explained an insignificant ($p > .05$) portion of the total variation in wheel running.

Over the 40-day experimental trial, C mice from Generation 25 ran between 1 and 6 km/day on running wheels. In S lines, the range spanned 2 to 18 km/day. The large individual variation in voluntary running within both C and S lines makes our model particularly useful for studying correlations between quantitative measures of physical activity and markers of neuronal plasticity.

BrdU-Positive Cell Number

BrdU-positive cells (mostly neurons; see Figures 2A and 2B, Table 1, and *Phenotypic Analysis of BrdU-Positive Cells* below) were counted in the granule cell layer of DG 30 days after the last injection of BrdU to measure neurogenesis (a combined result of proliferation, survival, and differentiation; Figures 2A and 2B, Table 1). Wheel running significantly increased BrdU-positive cell number, as indicated by a significant main effect of wheel access in the two-way ANOVA, $F(1, 86) = 505.1$, $p < .0001$ (Figures 2A, 2B, and 2C). Furthermore, the increase was greater in S mice than in C mice, as indicated by a significant interaction between wheel access and line type, $F(1, 86) = 7.7$, $p = .007$, and inspection of Figure 2C; no main effect of line type occurred, $F(1, 6) = 2.3$, $p = .18$. As indicated in Figure 2C, S runners had the highest number of BrdU-positive cells in DG, followed by C runners. S and C mice housed without wheels did not differ. Indeed, wheel running increased BrdU cell numbers 5-fold in S mice and 4-fold in C mice, as compared to mice without wheels (Figure 2C). Tukey's post hoc tests (to correct for multiple comparisons) support the interpretation that neurogenesis was greater in S mice than in C mice within the runners ($p = .04$), but not within the nonrunners ($p = .99$). Results were qualitatively similar when BrdU cell counts were adjusted for DG volume by including estimated DG volume as a covariate in the analysis (see Table 2).

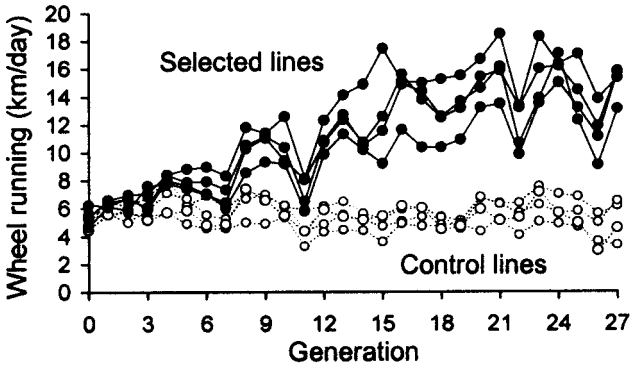

Figure 1. Twenty-seven generations of selective breeding produced four replicate lines of mice that display increased voluntary wheel-running behavior as compared with four random-bred control lines. Mean distance (in kilometers per day) run by females on Days 5 and 6 of a 6-day test are shown for each line, across generations, from the beginning of the selective breeding through Generation 27. Note that the differential has remained approximately constant since Generation 16.

Another interesting difference between C and S mice occurred in the correlation between running distance and BrdU-positive cell number. Specifically, the slope of the relationship between running distance and BrdU cells was steep for C mice but flat for S mice, as indicated by a significant interaction between running distance and line type, $F(1, 38) = 17.8$, $p < .0001$, and inspection of Figure 2D. Total number of BrdU-labeled cells in DG was positively correlated with running distance in C mice, $F(1, 18) = 13.1$, $p = .002$, consistent with previous studies of other mice (Allen et al., 2001). The correlation in C mice was strongest when wheel running was quantified as an average over the 10 days of BrdU injections but was statistically significant for all the wheel-running variables examined, including the average over the entire 40-day period. C mice that ran an average of 6 km/day had twice as many new neurons as did C mice that ran 1 km/day. In S mice, however, no relationship existed between distance run and BrdU cell number, $F(1, 18) = 0.8$, $p = .39$. S mice that ran an average of 18 km/day had the same high number of BrdU cells as S mice that ran 2 km/day (Figure 2D). Taken together, these results suggest that there may be a limit to the amount of neurogenesis that can occur with wheel running, and that all S mice reach this limit by virtue of their high levels of running.

Access to a running wheel increased DG volume by 20% in C mice, $F(1, 43) = 27.6$, $p < .0001$, and 17% in S mice, $F(1, 43) = 25.8$, $p < .0001$. We observed no differences between S and C lines, $F(1, 6) = 1.9$, $p = .21$, and no correlation with running distance when considering S and C mice together, $F(1, 39) = 1.5$, $p = .22$.

In a subsample of mice (C nonrunners, $n = 13$; C runners, $n = 6$; S nonrunners, $n = 9$; S runners, $n = 8$), we estimated the total number of granule neurons in DG. Without adjusting for DG volume, S mice had more granule neurons than C mice, $F(1, 6) = 5.7$, $p = .05$, and mice with access to running wheels tended to have more neurons than mice without wheel access, $F(1, 25) = 2.8$, $p = .11$; the interaction between wheel access and line type was not significant, $F(1, 25) = 0.02$, $p = .90$. Relative to the total number of granule neurons in C mice without wheels, C mice with wheels had 11% more cells, S mice without wheels had 13% more cells, and S mice with wheels had 23% more cells. When granule cell counts were adjusted for DG volume by including estimated DG volume as a covariate in the analysis, DG volume was a significant covariate, $F(1, 24) = 33.1$, $p < .0001$, and there

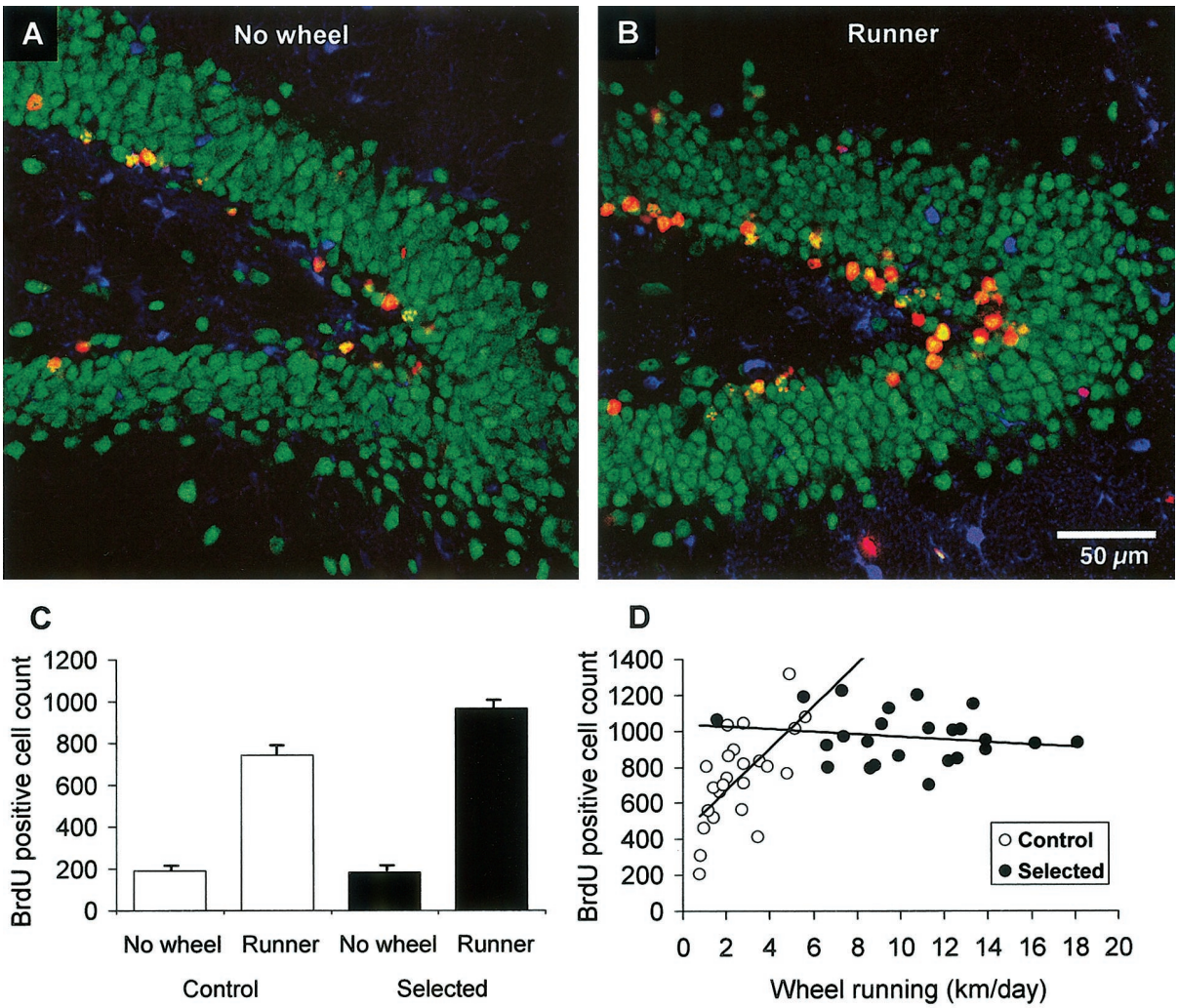

Figure 2. Wheel running increased neurogenesis more in mice selectively bred for increased voluntary wheel running (S) than in control (C) mice. A representative section through the dentate gyrus (DG) is shown for a mouse housed without a running wheel (Panel A) and with a running wheel (Panel B), indicating that a majority of the 5-bromodeoxyuridine (BrdU) cells in DG differentiated into neurons and that wheel running increased neurogenesis. Neurons are labeled with a green immunofluorescent marker (NeuN). BrdU nuclei are immunofluorescent red. Glial cells are immunofluorescent blue (S100β). Orange-yellow cells have both the green (NeuN), and red (BrdU) labels, indicating that they were born at the time the BrdU injections were administered (when mice were 30–40 days old) and subsequently matured into neurons (when mice were 70 days old). Purple cells have both the BrdU and S100β labels, indicating that they are newly formed glial cells. Panel C: Mean ($\pm$ *SEM*) number of BrdU-positive cells, counted in a series of sections 240 μm apart throughout DG of the hippocampus, indicating that neurogenesis increased more in S than in C runners. Panel D: The relationship between distance run (averaged over the 10 days mice were given BrdU injections) and BrdU cell number for C runners and S runners, indicating that neurogenesis is positively correlated with distance run in C runners but reaches a plateau in S runners.

were no longer any differences between groups: effect of line type, $F(1, 6) = 0.8$, $p = .42$; wheel treatment, $F(1, 24) = 0.2$, $p = .69$; and their interaction, $F(1, 24) = 0.1$, $p = .76$. This suggests that the number of granule neurons contributed to the larger DG volumes. DG volume, $F(1, 87) = 61.2$, $p < .0001$, and granule cell counts, $F(1, 27) = 7.6$, $p = .01$, were positively correlated with BrdU cell number (Figures 3A and 3B). Taken together, these results suggest that exercise-induced neurogenesis contributes cells to the granule layer and increases DG volume.

Phenotypic Analysis of BrdU-Positive Cells

Differentiation of the BrdU-positive cells was examined by immunofluorescent triple labeling for BrdU, the neuronal marker NeuN, and glial marker S100β. A subsample of mice (C nonrunners, $n = 10$; C runners, $n = 8$; S nonrunners, $n = 6$; S runners, $n = 7$) was used to determine the proportion of BrdU-positive cells expressing a neuronal phenotype. Thirty cells per mouse were analyzed by confocal microscopy (Zeiss, Bio-Rad). Wheel running

Table 2
Analysis of the Number of BrdU-Positive Cells: ANOVA Table and Least Square Adjusted Means (With 95% Confidence Intervals)

Term	Numerator *df*	Denominator *df*	*F*	*p*
Volume	1	85	7.76	.007
Wheel treatment	1	85	275.04	< .0001
Line type	1	6	0.87	.39
Line Type × Wheel Treatment	1	85	9.11	.003

Least square adjusted means

Control, no wheels	Control, wheels	Selected, no wheels	Selected, wheels
195.4 (151.1, 249.5)	669.5 (552.6, 805.3)	178.9 (139.3, 227.0)	887.8 (736.3, 1,063.2)

Note. Volume of the dentate gyrus (continuous variable), wheel treatment (factor with two levels: runners vs. nonrunners), line type (factor with two levels: selected vs. control) and the interaction (Line Type × Wheel Treatment) were entered as fixed effects. Line was entered as a random effect. The *F* and *p* values for the covariate (volume of the dentate gyrus) refer to a marginal test (Type 3), whereas *F* and *p* values for the factors refer to sequential tests (Type 1), and the terms were entered in the order that they appear in the table. Prior to statistical analyses, the numbers of BrdU-positive cells were transformed by raising to the exponent 0.2 to stabilize the variance between wheel treatments; otherwise, variance for runners was much greater than variance for nonrunners. The least square adjusted means were then transformed back to the original scale (and least square standard errors were converted to 95% confidence intervals); these values are shown in the table and were used for comparison and interpretation. The random effect, line, was significant, that is, the standard deviation of BrdU-positive cells, raised to the exponent 0.2, among lines within a line type, was significantly greater than zero, and was estimated to be 0.11, $\chi^2(1, N = 96) = 7.6$, $p = .006$. For the line effect, the chi-square test statistic was obtained by multiplying −2 by the difference in the restricted log likelihood for the model with versus without the random effect (likelihood ratio test; Littell et al., 1996; Pinheiro & Bates, 2000). BrdU = 5-bromodeoxyuridine.

increased the proportion of new cells differentiating into neurons, $F(1, 21) = 19.2$, $p = .0003$, and decreased the proportion of glia, $F(1, 21) = 13.4$, $p = .002$, and undifferentiated BrdU cells, $F(1, 21) = 8.8$, $p = .007$. S and C mice did not differ (Table 1). These findings suggest that a majority of the BrdU-labeled cells counted in the DG of this study became neurons in S and C mice as opposed to remaining undifferentiated or differentiating into glial cells (Figures 2A and 2B). Therefore, simply counting number of BrdU cells in DG is a measure of net neurogenesis (i.e., end result of proliferation, survival, and subsequent neuronal differentiation).

Hippocampal BDNF Concentration

Forty days of wheel access increased BDNF concentration in the hippocampus by 56% in C mice, $F(1, 24) = 11.1$, $p = .003$, and 38% in S mice, $F(1, 33) = 7.5$, $p = .01$ (see Figure 4). No differences in mean concentration of BDNF between the S and C mice occurred, $F(1, 5) = 0.2$, $p = .72$; nor was the interaction between wheel access and line type significant, $F(1, 57) = 0.6$, $p = .46$. No correlation between hippocampal BDNF concentration and distance run occurred, $F(1, 24) = 0.1$, $p = .72$, for S and C mice combined.

Morris Water Maze Learning

To assess spatial learning, mice were trained on a hidden-platform Morris water maze (Morris, 1984) with four trials per day for 6 days (following van Praag, Christie, et al., 1999). In an ANOVA with repeated measures (day), the interaction between wheel access and line type was statistically significant for latency, $F(1, 205) = 6.1$, $p = .01$, and path length, $F(1, 205) = 4.7$, $p = .03$, but not swim speed, $F(1, 205) = 0.02$, $p = .89$, indicating that access to a running wheel had a differential effect on learning in S versus C mice. In C mice, runners had decreased latency, $F(1, 80) = 5.1$, $p = .03$, and path length, $F(1, 80) = 4.2$, $p = .04$, compared with nonrunners (i.e., wheel access improved learning), consistent with previous studies (van Praag, Christie, et al., 1999). However, in S mice, no significant differences between the runners and nonrunners occurred for latency, $F(1, 110) = 2.1$, $p = .15$, or for path length, $F(1, 110) = 1.3$, $p = .25$. Moreover, the trend was in the opposite direction, with runners displaying increased latency and path length relative to nonrunners (Figure 5).

A comparison of learning curves between S and C mice might be complicated if S and C mice differed in latency or path length on Day 1 (Figure 5). However, there were no significant differences at this time point for latency, $F(1, 5) = 2.5$, $p = .18$, or for path length, $F(1, 5) = 0.9$, $p = .39$. Under baseline (no wheel) conditions, S and C mice displayed similar learning curves. For latency, day was significant, $F(5, 95) = 2.9$, $p = .02$, indicating that both S and C nonrunners learned, but the interaction between day and line type was not significant, $F(5, 95) = 0.2$, $p = .95$, indicating that learning curves were similar for S and C nonrunners For path length, day was also significant, $F(5, 95) = 3.1$, $p = .01$, but the interaction was not significant, $F(5, 95) = 0.4$, $p = .86$. Under conditions of wheel access, C mice displayed a steeper learning curve than S mice. For latency, the interaction between day and line type was significant, $F(5, 95) = 3.4$, $p = .008$, but path length, $F(5, 95) = 1.0$, $p = .42$, was not. However, latency, $F(5, 40) = 4.8$, $p = .002$, and path length, $F(5, 40) = 2.4$, $p = .056$, decreased (i.e., mice learned) with trial day in C runners, whereas latency, $F(5, 55) = 0.5$, $p = .81$, and path length, $F(5, 55) = 1.1$, $p = .35$, did not decrease with trial day in S runners (i.e., mice did not learn).

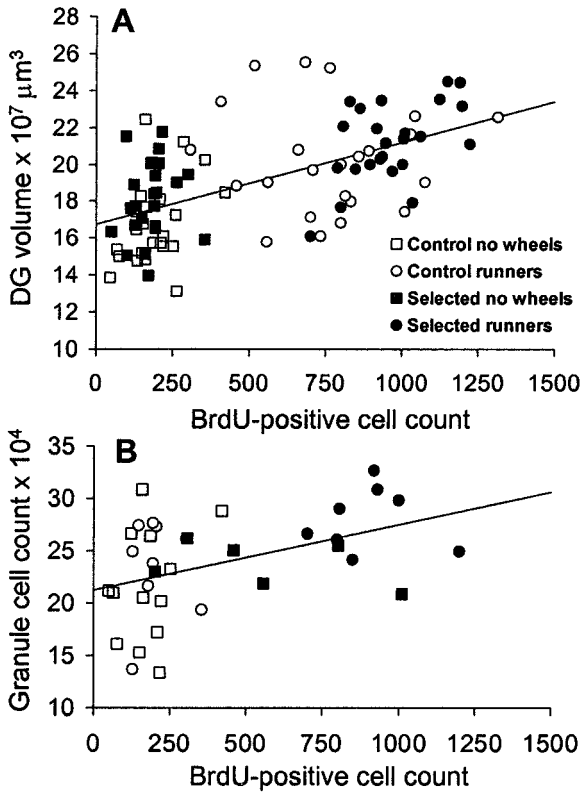

Figure 3. Neurogenesis predicts dentate gyrus (DG) volume and granule cell number. A: Estimated DG volume plotted against 5-bromodeoxyuridine (BrdU)-positive cell number fitted with simple linear regression. B: Estimated total number of granule cells plotted against BrdU-positive cell number fitted with simple linear regression. Estimates of DG volume and total number of granule neurons are consistent with Kempermann et al. (1997a).

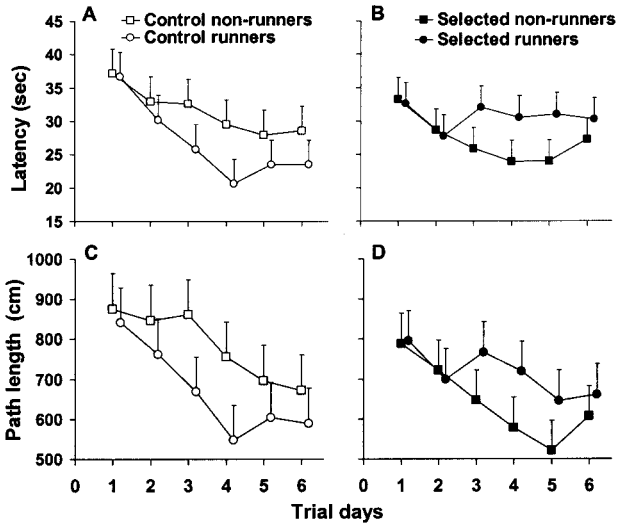

Figure 5. Access to a running wheel enhanced learning in control (C) mice, but not in mice selectively bred for increased voluntary wheel running (S). Mean (± *SEM*) latency to reach the hidden platform over 6 days of Morris water maze trials for (A) C runners and nonrunners and (B) S runners and nonrunners. Mean (± *SEM*) path length to reach the hidden platform for (C) C runners and nonrunners and (D) S runners and nonrunners. Individual values used to calculate the means are the average of four trials per day. Whereas wheel running improved learning in C mice (A, C; steeper learning curve for circles compared with squares), the pattern was reversed in S mice, with runners performing "worse" than nonrunners (B, D; steeper learning curve for squares compared with circles). All groups were shown to decrease latency and path length over days, except S runners, which failed to demonstrate learning.

The lack of improvement in learning in S runners versus S nonrunners appears specific to the spatial memory component of the Morris water maze task (Gerlai, McNamara, Williams, & Phillips, 2002), because there were no differences in learning between S and C mice with or without wheels when the platform was visible. In an ANOVA with repeated measures, day was significant for latency, $F(5, 205) = 6.7$, $p < .0001$, and path length, $F(5, 205) = 4.4$, $p = .0008$, indicating that the mice learned, but the interaction between wheel access and line type was not significant for latency, $F(1, 205) = 0.2$, $p = .63$, or for path length, $F(1, 205) = 0.8$, $p = .37$, indicating that there were no group differences in learning curves. Together with the hidden-platform results, these findings suggest that exercise enhanced spatial learning in C, but not S, mice.

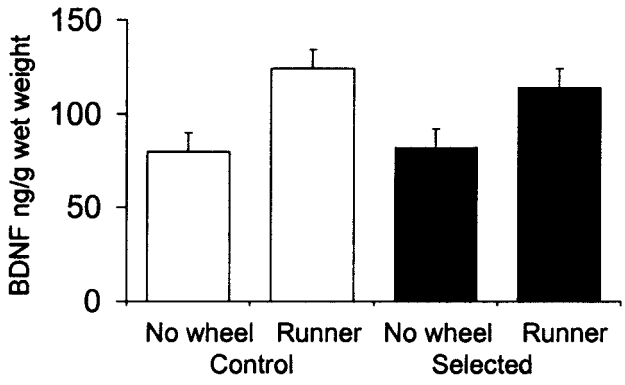

Figure 4. Wheel running increased brain-derived neurotrophic factor (BDNF) concentration by approximately 46% in both control mice and in mice selectively bred for increased voluntary wheel running. Bars represent mean (± *SEM*) concentration (in nanograms per gram wet weight) of BDNF in the hippocampus.

Discussion

Consistent with previous studies in rodents, access to a running wheel increased BDNF concentration (Neeper et al., 1995), hippocampal neurogenesis (van Praag, Christie, et al., 1999), and spatial learning (van Praag, Christie, et al., 1999) in normal C mice. In high-running S mice, access to a running wheel also increased BDNF concentration and neurogenesis. However, the positive correlation between neurogenesis and running distance was lost in S runners because of a possible ceiling effect for exercise-induced neurogenesis (Figure 2D). Thus, this is the first evidence that there may be a limit to the amount of neurogenesis that can occur with exercise. Another interesting result was that access to a running wheel did not improve spatial learning in S mice as it did in C mice. The basis for the lack of learning enhancement is unclear, as BDNF and neurogenesis were elevated

in S runners. It is possible that aberrant neural physiology associated with hyperactivity in S mice (Rhodes et al., 2001) disrupted cognitive function. On the other hand, it is also possible that the changes in BDNF and neurogenesis in both S and C mice were related to the possible role of the hippocampus in motor behavior (Morris & Hagan, 1983; Oddie & Bland, 1998) rather than learning or memory.

This study confirms that there is a strong relationship between exercise and neurogenesis (van Praag, Christie., et al., 1999). This is because neurogenesis was strongly correlated with running distance in the C mice of this study and in 129SvEv inbred mice (Allen et al., 2001). This is the first evidence that the correlation is lost for mice that exercise at extraordinarily high levels. The reason for the lost correlation is unclear. One possibility is that there is a limit to the number of new cells that can be produced in association with exercise, and that a plateau exists. Our data are consistent with a neurogenesis plateau occurring anywhere from the middle to the high end of the range in C mice. Alternatively, it is possible that the neural physiology of the S mice is aberrant such that normal mice, if they could be induced to exercise at higher levels, would show a continuous rise in neurogenesis with level of exercise.

The possibility that exercise might enhance learning is intriguing (Anderson et al., 2000; Fordyce & Wehner, 1993; van Praag, Christie, et al., 1999). The C runners displayed improved learning relative to the C nonrunners, confirming this relationship. On the other hand, the improved learning in the C runners versus the C nonrunners could be interpreted as an improvement over the deprived conditions of standard laboratory housing (P. B. Lavenex, Lavenex, & Clayton, 2001) rather than as an exercise enhancement (van Praag, Christie, et al., 1999). Thus, results in C mice might demonstrate either a connection between exercise and enhanced learning or a connection between environmental enrichment and enhanced learning (Kempermann et al., 1997b). If learning could be collapsed into one number (i.e., the 6-day learning curves could be summarized as one value), then, in theory, it would be possible to calculate a correlation between learning and level of exercise (e.g., distance run). Such a correlation would make it more convincing that these variables are functionally associated. On the other hand, if the relationship between exercise and learning is qualitative rather than quantitative, we might not expect to find a relationship between amount of running and learning. We considered using the difference in latency (Day 1 minus Day 6) and the first-order coefficient of a polynomial fit through the data (Day 1 through Day 6) as single measurements of learning. The latter measurement estimates the steepness of the learning curve at the hypothetical data point, Day 0. However, no correlation occurred between running distance and either single measure of learning (data not shown). Results in S mice demonstrate that high levels of exercise are not beneficial for learning, which suggests that exercise itself may not be causally related to the learning improvement in normal mice. On the other hand, if the S mice are hyperactive (Rhodes et al., 2001), then aberrant neural physiology related to their hyperactivity might interfere with their learning abilities.

The mechanism behind the lack of improvement in spatial learning in S runners is not clear. Several characteristics known to differ between S and C mice might have contributed to the learning differences. The S mice are hyperactive (Rhodes et al., 2001), and their hyperactivity might have caused problems in attention to complex cues. On the basis of their responses to dopamine drugs, it has been suggested that S mice have altered dopaminergic function (Rhodes et al., 2001; Rhodes & Garland, 2003). Because dopamine receptor blockade in the nucleus accumbens impairs spatial learning in rats (Ploeger, Spruijt, & Cools, 1994), differences in dopaminergic modulation of the hippocampus or hippocampal outputs such as the nucleus accumbens (Bardgett & Henry, 1999; Brudzynski & Gibson, 1997; Floresco, Todd, & Grace, 2001; Wu & Brudzynski, 1995) might have affected spatial learning in S mice. S runners also have elevated corticosterone levels (Girard & Garland, 2002; Sapolsky, 1996), and corticosterone can interfere with learning (Sapolsky, 1996). It is also possible that differences in the estrous cycle (which was not measured) between S and C mice might affect spatial learning (Warren & Juraska, 1997), although no data exists to support a difference in estrous cycling between S and C runners at this time.

Exercise-induced changes in BDNF protein levels could influence hippocampal neurogenesis and spatial learning (Lee et al., 2000; Pencea et al., 2001). Consistent with earlier reports (Neeper et al., 1995) of increased BDNF mRNA expression after wheel running, exercise increased BDNF protein levels in the hippocampus of both S and C mice. In contrast to earlier studies (Neeper et al., 1995; Oliff, Berchtold, Isackson, & Cotman, 1998), we did not find a correlation between BDNF levels and running distance. This difference may be attributable to the measured variable (mRNA vs. protein) or to the fact that we measured BDNF concentration after 40 days of running versus 1–7 days, and in females rather than males (Neeper et al., 1995; Oliff et al., 1998). A positive correlation was observed between hippocampal BDNF and running distance in male S and C mice given 7 days of running wheel access (Johnson, Rhodes, Jeffery, Garland, & Mitchell, in press). BDNF levels during this early period might have contributed to the greater neurogenesis in female S runners relative to C runners. After 40 days, BDNF levels were similar between S and C runners even though neurogenesis was greater in S than C runners. Because learning was impaired in the S runners and because BDNF did not increase in proportion to the numbers of new neurons at 40 days, one possible explanation for the lack of learning in S runners is that there was inadequate trophic support for newly generated cells in S runners, leading to impaired function (Black, 1999). This suggestion leaves open the possibility of diminished survival (Li, Jarvis, Alvarez-Borda, Lim, & Nottebohm, 2000) or functional integration (van Praag et al., 2002) of new neurons in S runners.

The possibility that exercise-induced neurogenesis plays a functional role in learning is an intriguing hypothesis. However, strong data in support of this hypothesis do not exist at this time. Consistent with the learning hypothesis is that C mice and C57BL/6 inbred mice (van Praag, Christie, et al., 1999) both show increased neurogenesis and enhanced learning when given access to a running wheel. However, these variables might be correlated without being causally related. Feng et al. (2001) demonstrated that presenelin-1 knockout mice learn as well as wild-type mice on a hippocampus-dependent task even though they show deficient neurogenesis. Feng et al. (2001) concluded that neurogenesis and hippocampal learning can be dissociated. Therefore, we considered an alternative functional explanation for exercise-induced neurogenesis: that it might play a role directly related to motor behavior. A role for exercise-induced neurogenesis in motor behavior has never previously been proposed. The feasibility of this hypothesis depends on whether the hippocampus plays a role in motor behavior, and whether the hippocampus has a limited ca-

pacity to generate the motor behavior that might require the addition of new neurons.

Although the hippocampus is most well known for its role in learning and memory, several studies have identified roles in motor behavior (Bardgett & Henry, 1999; McFarland, Teitelbaum, & Hedges, 1975; McNaughton, Barnes, & O'Keefe, 1983; Morris & Hagan, 1983; Oddie & Bland, 1998; Vanderwolf, 1969). The supposition that the hippocampus has a motor function has been questioned on the basis of evidence that its destruction does not prevent locomotion (as reviewed in Oddie & Bland, 1998). However, destruction of the hippocampus changes qualitative aspects of movement execution, such as the intensity at which a motor act is carried out (Morris & Hagan, 1983; Oddie & Bland, 1998). For example, although hippocampal lesions do not prevent rats from jumping (Myhrer, 1975), they reduce the height at which rats are capable of jumping (Oddie & Bland, 1998). Moreover, the frequency of theta waves in the hippocampus increases immediately (on the order of 100 ms) preceding a jump, and the length of the period of the theta wave coinciding with take-off is strongly, inversely correlated with the height, velocity, and peak force parameters of the jump (Morris & Hagan, 1983). Theta activity in the rat hippocampus is necessary to induce spontaneous wheel running behavior through electrical stimulation of the posterior hypothalamus (Oddie & Bland, 1998), and the frequency of this hippocampal theta (adjusted by the intensity of electrical stimulation) is closely correlated with wheel-running speed (Slawinska & Kasicki, 1998). Thus, although rodents may be able to move without a hippocampus, they cannot produce the intense movements typically associated with various types of exercise (e.g., high jumps or wheel running behavior).

If exercise-induced neurogenesis were related to motor behavior, then the hippocampus should display some limited capacity to regulate the motor behavior that requires the addition of new neurons. Moreover, if the new neurons enhance the capacity for exercise performance, then the enhanced performance must lag behind the addition of the new neurons. Wheel running behavior increases gradually during the first 20 days of wheel exposure (for the mice in this study, wheel running increased by approximately 0.63 km/day for S mice and 0.18 km/day for C mice during the first 20 days; see also Swallow, Koteja, Carter, & Garland, 1999). Therefore, it is possible that the addition of new neurons in DG facilitated the rise in exercise levels over the 20-day period in both S and C mice. Moreover, in a separate study (Rhodes, Garland, & Gammie, in press), we found that neuronal activity (as measured by Fos immunohistochemistry) in DG was positively correlated with distance run in C mice but reached a high plateau in S mice, similar to neurogenesis (Figure 2D). Therefore, it is possible that new neurons are needed to extend the capacity of DG to regulate intense bouts of exercise. The new cells may also balance a possible increase in cell death (Biebl, Cooper, Winkler, & Kuhn, 2000) that occurs in DG in response to exercise-induced neuronal activation. The possibility that a plateau exists for this process is consistent with our inability to produce greater levels of wheel-running exercise in S mice since approximately Generation 16 (i.e., a selection limit has been reached, see Figure 1; see also Garland, 2003).

The present study confirms that access to a running wheel increases hippocampal BDNF concentration (Neeper et al., 1995), hippocampal neurogenesis (van Praag, Kempermann, & Gage, 1999), and spatial learning (Anderson et al., 2000) in normal rodents. The study also confirms that there is a strong positive correlation between running distance and neurogenesis in normal mice (Allen et al., 2001). Results for S mice provide the first evidence that there may be a limit to exercise-induced neurogenesis because the correlation between running distance and neurogenesis was lost in S mice as a result of a possible ceiling effect (Figure 3D). Another noteworthy finding was that despite extraordinarily high levels of exercise, and high levels of exercise-induced neurogenesis in S mice, spatial learning was not improved. The possibility that neurogenesis represents a mechanism for learning is exciting, but future work will be needed to resolve this issue. In the meantime, we suggest it is useful to consider alternative possibilities regarding the functional significance of exercise-induced neurogenesis, such as a role in locomotor performance.

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