Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus

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Exposure to an enriched environment increases neurogenesis in the dentate gyrus of adult rodents. Environmental enrichment, however, typically consists of many components, such as expanded learning opportunities, increased social interaction, more physical activity and larger housing. We attempted to separate components by assigning adult mice to various conditions: water-maze learning (learner), swim-time-yoked control (swimmer), voluntary wheel running (runner), and enriched (enriched) and standard housing (control) groups. Neither maze training nor yoked swimming had any effect on bromodeoxyuridine (BrdU)-positive cell number. However, running doubled the number of surviving newborn cells, in amounts similar to enrichment conditions. Our findings demonstrate that voluntary exercise is sufficient for enhanced neurogenesis in the adult mouse dentate gyrus.

Brain diseases such as Alzheimer's[1] or Parkinson's[2] and injury such as stroke[3] have been considered to result in permanent loss of neurons with no possibility of cellular regeneration. This widely held belief has been challenged recently by extensive evidence that certain brain areas retain the capability to generate new neurons into adulthood in rodents[4–8], nonhuman primates[9] and humans[10]. The mechanisms by which these new neurons are generated and could contribute to brain repair are poorly understood. Recent studies indicate that exposure to an enriched environment[11] produces not only a host of structural and functional changes in the brain[12–14], but also a significant increase in hippocampal neurogenesis[6]. Enrichment, however, is a complex combination of inanimate and social stimulation[15], consisting of larger housing and more opportunity for social interaction, physical activity and learning than standard laboratory living conditions. It is not known which of these factors is critical for fostering survival of newborn dentate gyrus granule cells. Here we separated out components of the enriched environment and studied their effects on adult hippocampal cell proliferation and neurogenesis.

Enhanced neurogenesis in enriched animals has been associated with improved spatial memory performance[6,16,17]. Conversely, learning itself may be a specific stimulus for neurogenesis. Maze training and enrichment may result in similar neurochemical alterations[18]. Moreover, in food-storing birds, storage and retrieval experiences are correlated with changes in hippocampal size and neurogenesis[19–21]. An important confounding variable in assessing the immediate effects of learning on adult hippocampal neurogenesis is motor activity, which could affect cell proliferation, survival or differentiation. Indeed, exercise facilitates recovery from brain injury such as stroke[22] and enhances cognitive function[23,24]. Moreover, physical activity enhances neurotrophin levels and gene expression[25]. In particular, the level of basic fibroblast growth factor (bFGF), which is important for the survival and differentiation of progenitor cells *in vitro*[26,27] and *in vivo*[28,29], is elevated by exercise as well as by spatial learning[30,31]. We designed our study to investigate the contribution of these variables, learning and physical activity, to generation of new dentate granule cells. Thus, we assigned mice to enriched-environment, hidden-platform water-maze learning, forced-exercise (yoked-swim controls), voluntary-exercise (running wheel) or standard-living (control) conditions.

We show that neither water-maze training nor yoked swimming had any effect on cell proliferation or neurogenesis. Exposure to an enriched environment increased the number of surviving newborn cells but did not affect proliferation, confirming our previous studies in C57BL/6 mice[6,16]. Voluntary exercise in a running wheel increased cell proliferation, cell survival and net neurogenesis. Our findings suggest that physical activity is sufficient to enhance several aspects of adult hippocampal neurogenesis.

RESULTS

Proliferation and survival of BrdU-labeled dentate cells

Subgranular progenitor cell proliferation was addressed by BrdU labeling of dividing cells over 12 days and immunohistochemical analysis one day after the last injection. A significant difference was found between the groups ($F_{4,25} = 4.32$, $p < 0.01$). Specific comparisons showed that the runners had more proliferation than any of the other groups examined ($p < 0.02$; **Fig. 1a**).

Survival of the progeny of the dividing progenitor cells was assessed by staining for BrdU-positive cells four weeks after the last injection of BrdU. In control and learner groups, 42% of the proliferating cells survived, in swimmers, 46% and in runners, 56%. Significantly more relative survival was observed in the enriched group, 85%, than in any other groups ($F_{4,35} = 3.86$, $p < 0.01$). Furthermore, a highly significant difference in the number of labeled cells was found between the groups ($F_{4,35} = 6.12$, $p < 0.0009$). The total number of surviving cells was significantly greater in runner

Fig. 1. BrdU-positive cell number. **(a)** Total number of BrdU-positive cells per dentate gyrus one day after the last BrdU injection, to estimate ongoing proliferation. Significantly more cells were labeled in the runners as compared to the other groups. $*p < 0.02$. **(b)** Total number of BrdU-positive cells per dentate gyrus four weeks after the last BrdU injection, to estimate survival of labeled cells. Enrichment and running significantly increased the survival of newborn cells. $*p < 0.02$.
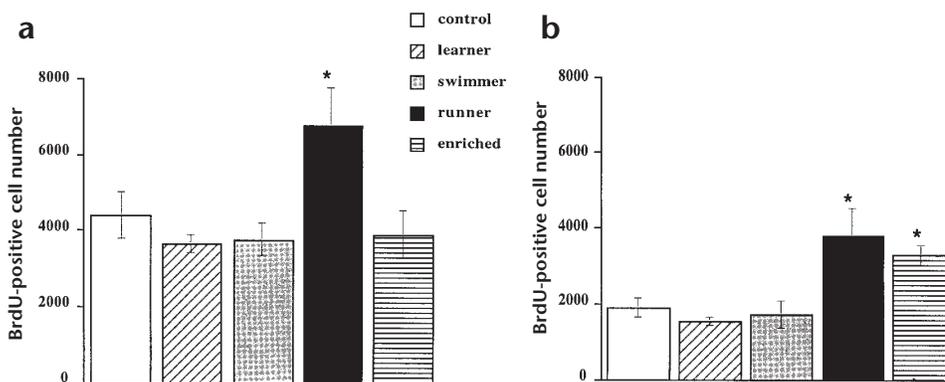
($p < 0.002$) and enriched ($p < 0.02$) groups than in controls, learners or swimmers. Runners and enriched mice had 201% and 175%, respectively, of control levels of labeled cells per dentate gyrus (**Fig. 1b**). The volume of the dentate gyrus, however, did not differ between the groups ($F_{4,35} = 1.06$, $p > 0.39$; **Table 1**).

Differentiation of the surviving BrdU-positive cells was examined four weeks after the last BrdU injection by immunofluorescent triple labeling for BrdU, neuronal marker NeuN[32] and glial marker S100b[33]. Runners and enriched animals differed significantly from controls ($p < 0.02$) and swimmers ($p < 0.001$) with regard to the percentage of cells labeled for NeuN ($F_{4,35} = 5.79$, $p < 0.001$) and of cells labeled for neither NeuN nor S100b, ('other'; $F_{4,35} = 5.54$, $p < 0.0015$). There was no significant difference between the groups with regard to the percentage of newborn cells that differentiated into glia ($F_{4,35} = 2.21$, $p > 0.08$; **Fig. 2; Table 1**).

Water-maze training

Learners were trained to find the hidden platform in the Morris water maze[34]. We used blocks of two trials each day so that the task would remain challenging over the four-week training period. Initially, 14 mice were trained over 12 days, during which latency to reach the platform decreased significantly ($F_{13,11} = 6.17$, $p < 0.0001$). On day 13, 6 of these animals were perfused to assess cell proliferation. The remaining 8 mice were trained for an additional 11 days, and showed a significant reduction in time needed to reach the platform ($F_{7,22} = 3.51$, $p < 0.0001$). Thereafter, platform

location was changed for seven days of reversal training. Escape latency decreased significantly over 7 days ($F_{7,6} = 9.98$, $p < 0.0001$; **Fig. 3**). Trial duration for the swimmers was the average learner latency on each training day. Our morphological results show, however, that neither proliferation (at day 13, one day after the last BrdU injection) nor cell survival (at day 43, four weeks after the last BrdU injection) was affected in the learner or swimmer groups. These findings suggest that neither extensive training in this spatial learning task nor limited forced exercise affects proliferation or survival of BrdU-positive cells in the dentate gyrus.

DISCUSSION

The present study was designed to determine whether physical activity, be it voluntary, forced or in combination with a learning task, would be involved in the enhanced adult hippocampal neurogenesis observed following exposure to enriched environments[6,16,17]. The results show that cell proliferation was only increased in mice housed with unrestricted access to a running wheel (runners). Moreover, both voluntary physical activity and enrichment roughly doubled the total number of surviving newborn cells in the dentate gyrus. In addition, in both the enriched and runner groups, relatively more BrdU-positive cells exhibited neuronal phenotype. In contrast, mice trained in the water maze and yoked-swim controls showed no change in BrdU-positive cell number, suggesting that this type of learning or activity alone is not an adequate stimulus for adult hippocampal neurogenesis.

Our results also allowed us to exclude several other factors that could affect newborn hippocampal cells. Neither enriched nor runner mice received any treats such as apples or cheese, which had been given as part of the enrichment protocol in our earlier studies[6], ruling out diet as a possible confounding factor. Furthermore, running mice were housed in groups of three or four, suggesting that interaction with a large social group[15] is not necessary to elicit enhanced cell survival. It is possible, though, that the social factor is involved in the relatively higher percentage of surviving BrdU-positive cells in enriched versus runner mice. Finally, in contrast to our previous experiments in which BrdU labeling began after a month[6,16,17], injections were given

Table 1. Proliferation, survival and phenotypes of BrdU-positive cells.

	Control	Learner	Swimmer	Runner	Enriched
Proliferation, 1 day	4393 (607)	3637 (498)	3755 (422)	6773 (971)***	3867 (617)
Survival, 4 weeks	1880 (251)	1529 (120)	1711 (352)	3791 (715)**	3282 (265)**
Survival (%), 4 weeks	43 (5.7)	42 (12.8)	46 (6)	56 (10.6)	85 (6.8)***
Phenotypes:					
Neuron (%)	76.8 (3.2)	82.5 (2.6)	73.8 (3.6)	88.3 (1)*	88.8 (2.7)*
Astrocyte (%)	7 (2.1)	3.8 (2.7)	6.3 (0.8)	3.3 (0.6)	3 (1.1)
Other (%)	16.3 (1.7)	13.8 (2.1)	20 (3)	9 (1.3)*	8.3 (2)*
Volume (mm³)	0.43 (0.02)	0.37 (0.03)	0.34 (0.03)	0.42 (0.02)	0.34 (0.03)

C57BL/6 mice ($n = 70$) were assigned to control, learner, swimmer, runner or enriched groups. All mice received BrdU (50 mg per g) from day 1 to day 12. Cell proliferation was assessed on day 13 ($n = 6$ per group), 1 day after the last injection. Survival of BrdU-labeled cells and volume of the dentate gyrus were determined four weeks after the last BrdU injection ($n = 8$ per group). Phenotypes of the surviving cells were determined by immunofluorescent triple labeling for BrdU, NeuN (neurons) and S100b (astrocytes). The percentages of BrdU-positive cells double labeled for either NeuN or S100b or neither marker are presented. All data are presented as means (standard error). ***Significantly different from all other groups ($p < 0.02$). **Significantly different from controls, learners and swimmers ($p < 0.02$). *Significantly different from controls and swimmers ($p < 0.02$).
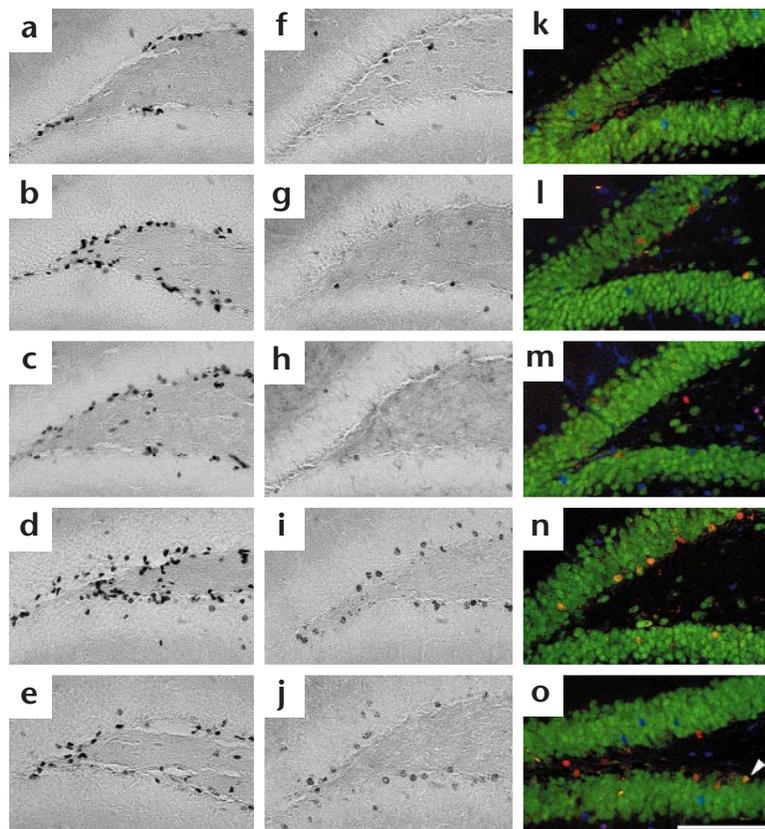
Fig. 2. Proliferation and neurogenesis in the dentate gyrus. Photomicrographs of BrdU-positive cells one day **(a–e)** and four weeks **(f–j)** after the last injection in control (a, f), learner (b, g), swimmer (c, h), runner (d, i) and enriched (e, j) mice. Confocal images of BrdU-positive cells in control (k), learner (l), swimmer (m), runner (n) and enriched groups (o), four weeks after the last injection. Sections were immunofluorescent triple labeled for BrdU (red), NeuN indicating neuronal phenotype (green) and S100b selective for glial phenotype (blue). Arrow in (O) shows BrdU-labeled neurons. (Orange is red plus green.) Scale bar, 100 µm.

erated cells was lower in runner (56%) than in enriched (85%) mice, suggesting differential long-term effects of these behavioral protocols.

Locomotion is highly correlated with the hippocampal theta rhythm[40]. Mice usually make heavy use of their running wheel, going about 20,000–40,000 revolutions per day[41]. Indeed, prolonged, locomotion-induced, synchronous electroencephalogram activity may alter neurochemistry. In turn, changes in neurotransmitter function may cause subtle, but important, changes in theta-rhythm frequency. For example, changes in serotonergic transmission can shift theta-rhythm frequency upward[42,43], enhance long-term potentiation as well as memory function[42], and possibly affect production of newborn granule cells (B.L. Jacobs, P. Tanapat, A.J. Reeves & E. Gould, *Soc. Neurosci. Abstr.* **24**, 796.6, 1998). In contrast, the length of the period of forced locomotion in our swimming tasks (approximately between 12 and 40 s per day) may be too short to cause long-lasting changes. Alternatively, these tasks may cause stress[9,44], counterbalancing the possible effects of activity on survival of BrdU-positive cells.

Maze training may evoke neurochemical events similar to those observed under enrichment conditions[18]. However, in our study, basal rates of proliferation and neurogenesis did not change after one month of training in the Morris water maze. It is possible that two trials per day did not provide sufficient exposure
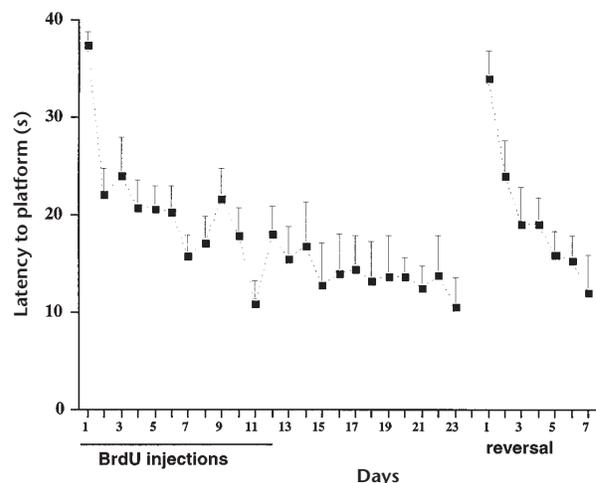
from the day of housing in each of the respective conditions, suggesting that effects on neurogenesis are relatively rapid.

Enriched living and running-wheel exercise resulted in approximately equal numbers of surviving BrdU-positive cells. In addition, in both protocols, relatively more cells became neurons than in controls. Furthermore, the percentage of cells that stained for neither glial nor neuronal markers was reduced. This group of cells may contain mature cells with phenotypes not examined here, neurons or astrocytes, in the process of differentiation before expression of specific markers, or neural and glial progenitor cells. It is possible that these progenitors are derived from a pool of multipotent hippocampal stem cells[35], whose fate could be influenced similarly by enriched experience and running. Neurochemical markers such as acetylcholine[36,37] and trophic factors, such as nerve growth factor and brain-derived neurotrophic factor[25,38], are affected by exercise and enrichment. However, these factors are elevated in enriched conditions only after additional exposure to behavioral tests[36,38], suggesting the existence of differential mechanisms. Moreover, enriched living had no effect on proliferation in C57BL/6J mice[6,16], whereas running did increase the number of BrdU-positive cells at one day after the last BrdU injection in this strain. Interestingly, in 129/SvJ mice, exposure to an enriched environment does increase BrdU-positive cell number at one day[17], suggesting different genetic bases for cell proliferation. Our present study shows that, within a constant genetic background, cell proliferation and survival can be controlled by different behavioral manipulations. Thus, one type of very focused activity, running, may have shortened the cell cycle (see also ref. 39) or induced additional quiescent cells to enter the cell cycle. Moreover, although runner and enriched mice had comparable numbers of BrdU-positive cells four weeks after the last injection, in relative terms the survival of newly gen-

Fig. 3. Water-maze training in the learners group. Latency to the platform became significantly shorter over 23 days as well as after 7 days of platform reversal ($p < 0.0001$).
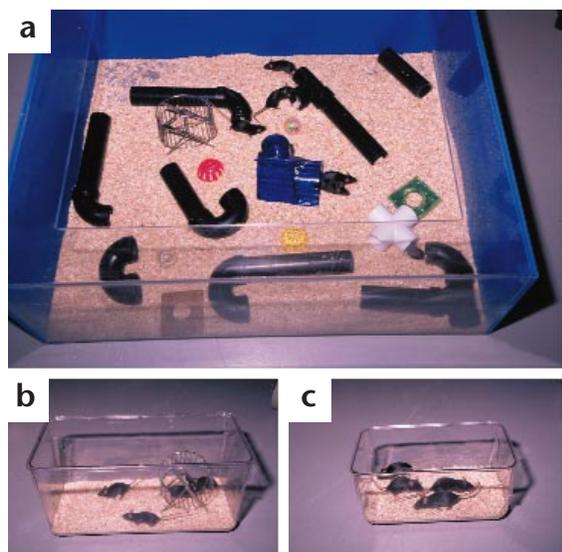
Fig. 4. Living conditions in the different experimental groups. **(a)** Cage for an enriched environment (86 x 76 cm). Enrichment consisted of social interaction (14 mice per cage), stimulation of exploratory behavior with objects such as toys and a rearrangeable set of tunnels and running wheels for exercise. **(b)** Cage containing running wheel for voluntary physical exercise (48 x 26 cm). **(c)** Standard housing cage (30 x 18 cm).

to the task to elicit an effect. Short-term massed training, which induces a transient increase in hippocampal bFGF mRNA[31], may be more effective. Indeed, another report in this issue shows that Morris water maze training at four trials per day over four days in rats increases the number of surviving BrdU-positive cells[45]. It is noteworthy, though, that some manipulations that increase neurogenesis are not necessarily compatible with learning. Blockade of NMDA receptors, which are normally required for learning[46], increases adult neurogenesis[47]. In addition, adrenalectomy impairs memory function[48] but elicits cell division in the dentate gyrus[49]. Furthermore, pathological events such as seizures have been reported to stimulate proliferation and neurogenesis[50]. Thus, upregulation of neurogenesis may be a rather general phenomenon, possibly increasing hippocampal storage capacity, whereas a specific learning task may influence existing cells.

In summary, our results demonstrate that voluntary exercise results in increased cell proliferation, survival and neuronal differentiation in the hippocampus of adult mice.

METHODS

Housing conditions. Seventy female C57BL/6 mice, three months old, were obtained from Jackson Laboratories (Bar Harbor). Animals were divided into 5 groups of 14 mice each: controls, learners, swimmers, runners and enriched. Learners received daily training in the Morris water maze, whereas swimmers were placed in the water maze for the same amount of time without a task (see below). The controls, learners and swimmers were placed in standard cages, with 3 or 4 animals per cage; the runners lived in rat cages with 1 running wheel, with 3 or 4 animals per cage; 14 mice were housed together in an enriched environment (see **Fig. 4**). The enriched environment was similar to our previous studies[6,16,17], except that animals did not receive dietary supplements or treats such as cheese and apples. During the first 12 days, animals received BrdU injections (see below). On day 13, 6 animals from each group were given an overdose of anesthetics and perfused transcardially with cold 4% paraformaldehyde in 0.1 M PBS. The remaining animals, eight per group,

continued in their respective experimental conditions for 29 days. On day 43, the remaining mice were perfused.

BrdU injections. BrdU (Sigma, St. Louis, Missouri) was dissolved in 0.9% NaCl and filtered sterile at 0.2 mm. The mice received single doses of 50 µg/g body weight at a concentration of 10 mg/ml, 1 intraperitoneal injection per day for 12 consecutive days.

Water-maze training. Learners were tested with 2 trials per day over 30 days. The platform was hidden 1 cm below the surface of water made opaque with white nontoxic paint. The two starting points were changed daily. Platform location was constant for 23 days. Thereafter, mice were trained for seven days with the platform in the opposite quadrant. Each trial lasted either until the mouse had found the platform or for a maximum of 40 s. All mice were allowed to rest on the platform for 10 s. Time to reach the platform (latency), length of swim path and swim speed were recorded semiautomatically by a video tracking system (San Diego Instruments). Swimmers were placed in the pool without the platform for two trials per day for 30 days. Trial duration for swimmers was the average learner group latency on that same day.

Immunohistochemistry. Immunohistochemistry for BrdU and immunofluorescent triple labeling for BrdU, NeuN, and S100b were done as described[29]. All staining was done on free-floating 40-µm sections that were pretreated for BrdU immunohistochemistry by denaturing DNA. The antibodies used were mouse anti-BrdU (Boehringer Mannheim, Indianapolis, Indiana) 1:400; rat anti-BrdU ascites (Accurate, Harlan Sera-Lab, Loughborough, England; for triple labeling), 1:100; rabbit anti S100b (Swant, Bellinoza, Switzerland) 1:2500; and mouse anti-NeuN (kindly provided by R.J. Mullen, University of Utah, Salt Lake City, Utah), 1:20. To determine the number of BrdU-labeled cells, we stained for BrdU with the peroxidase method (ABC system, with biotinylated donkey anti-mouse antibodies and diaminobenzadine as chromogen; Vector Laboratories, Burlingame, California). The fluorescent secondary antibodies used were anti-mouse FITC, anti-rat Texas Red, and anti-rabbit Cy5 (Jackson ImmunoResearch, West Grove, Pennsylvania), 6 µl/ml.

Analysis of phenotypes. A one-in-twelve series of sections from animals surviving four weeks after the last injection of BrdU was triple-labeled as described above and analyzed by confocal microscopy (Zeiss, Bio-Rad, Richmond, California). Fifty BrdU-positive cells per animal were analyzed for co-expression of BrdU and NeuN for neuronal phenotype and S100b for glial phenotype. Ratios of BrdU-positive cells colabeling with NeuN, with S100b or with neither NeuN nor S100b were determined.

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

Statistical analyses. ANOVA was used for morphological data. ANOVA with repeated measures over days was applied to the behavioral data. Specific comparisons were made with Fisher's post-hoc test.

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