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INTACT NEUROGENESIS IS REQUIRED FOR BENEFITS OF EXERCISE ON SPATIAL MEMORY BUT NOT MOTOR PERFORMANCE OR CONTEXTUAL FEAR CONDITIONING IN C57BL/6J MICE

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Abstract—The mammalian hippocampus continues to generate new neurons throughout life. Experiences such as exercise, anti-depressants, and stress regulate levels of neurogenesis. Exercise increases adult hippocampal neurogenesis and enhances behavioral performance on rotarod, contextual fear and water maze in rodents. To directly test whether intact neurogenesis is required for gains in behavioral performance from exercise in C57BL/6J mice, neurogenesis was reduced using focal gamma irradiation (3 sessions of 5 Gy). Two months after treatment, mice (total n=42 males and 42 females) (Irradiated or Sham), were placed with or without running wheels (Runner or Sedentary) for 54 days. The first 10 days mice received daily injections of bromodeoxyuridine (BrdU) to label dividing cells. The last 14 days mice were tested on water maze (two trials per day for 5 days, then 1 h later probe test), rotarod (four trials per day for 3 days), and contextual fear conditioning (2 days), then measured for neurogenesis using immunohistochemical detection of BrdU and neuronal nuclear protein (NeuN) mature neuronal marker. Consistent with previous studies, in Sham animals, running increased neurogenesis fourfold and gains in performance were observed for the water maze (spatial learning and memory), rotarod (motor performance), and contextual fear (conditioning). These positive results provided the reference to determine whether gains in performance were blocked by irradiation. Irradiation reduced neurogenesis by 50% in both groups, Runner and Sedentary. Irradiation did not affect running or baseline performance on any task. Minimal changes in microglia associated with inflammation (using immunohistochemical detection of cd68) were detected at the time of behavioral testing. Irradiation did not reduce gains in performance on rotarod or contextual fear, however it eliminated gain in performance on the water maze. Results support the hypothesis that intact exercise-induced hippocampal neurogenesis is required for improved spatial memory, but not motor performance or contextual fear in C57BL/6J mice. Published by Elsevier Ltd on behalf of IBRO.

Key words: exercise, neurogenesis, learning, irradiation, hippocampus, mice.

The recent discovery that physical exercise can benefit cognitive performance in humans has generated much enthusiasm and interest (Kramer et al., 2006). If exercise represents a natural generator of plasticity, then elucidating mechanisms of the natural generator has promise for enhancing cognition or combating cognitive decline from aging, stroke, trauma, or neurodegenerative disease. As compared with our understanding of the biology underlying how exercise enhances physical health (e.g., strength and stamina; Martin, 1987), the biology responsible for benefits in the brain are relatively unknown.

Exercise appears to benefit a range of cognitive abilities in animals and humans including spatial memory, working memory, executive control, and processing speed (Colcombe and Kramer, 2003; Van der Borght et al., 2007). Both the animal and human literature has established a number of changes in the nervous system that are correlated with exercise, some of which have been suggested to contribute to cognitive gain. These include changes in blood flow (Holschneider et al., 2007), concentrations of neurotransmitters (Meeusen and De Meirleir, 1995), growth factors (Cotman et al., 2007), trophic factors (Neeper et al., 1995), angiogenesis (Swain et al., 2003), gliagenesis (Li et al., 2005), and neurogenesis (van Praag et al., 1999a). Any or all of these changes could contribute to enhanced performance on a given task. Despite the abundance of correlative evidence, few studies have directly tested whether any of these changes play a functional role in enhancing cognition from exercise on any particular task.

One brain function that has repeatedly shown benefits from exercise is spatial learning and memory (van Praag et al., 1999b; Anderson et al., 2000; Van der Borght et al., 2007), the ability to remember the location of an object relative to other objects in the environment. Spatial memory is particularly sensitive to lesions in the hippocampus (Cho et al., 1999). This is consistent with growing knowledge of the role for the hippocampus in processing spatial or "place" information, and discovery of "place" cells in the hippocampus (O'Keefe and Dostrovsky, 1971). The dentate gyrus of the hippocampus is also one of the few regions in the adult mammalian brain that continues to generate new neurons throughout life (Altman and Das,

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Abbreviations: BrdU, bromodeoxyuridine; NeuN, neuronal nuclear protein; PBS, phosphate buffer solution; TBS, tissue buffering solution.

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1965). Moreover, exercise massively increases rate of hippocampal neurogenesis and these changes are associated with increased volume of dentate gyrus, total number of granular neurons, and increased long term potentiation (van Praag et al., 1999b; Rhodes et al., 2003b). The aim of this study was to directly test whether intact neurogenesis is required for enhanced spatial performance from exercise in C57BL/6J mice. This constitutes a test of the hypothesis that the correlation between number of new neurons and enhanced memory from exercise observed for this strain in previous studies (van Praag et al., 1999b, 2005) is due, in part, to a causal relationship.

We used focal gamma irradiation directed at the hippocampal region via a lead shield/stereotaxic apparatus to interfere with neurogenesis. The goal was to observe whether or not an animal with reduced capacity for neurogenesis is still capable of demonstrating improved behavioral performance on tasks that show gains from exercise in intact animals. Irradiation is a relatively new tool to reduce populations of rapidly dividing or undifferentiated neuronal precursors with minimal damage to fully differentiated neurons, glia or endothelial cells (Wojtowicz, 2006). In our study, the lead shield collimated radiation to a defined region of the brain around the hippocampus sparing many other regions including prefrontal cortex, caudate, cerebellum, hindbrain and spinal cord. All these features contributed to specificity of the radiation method for targeting hippocampal neurogenesis in this study. Nonetheless, it is known that irradiation induces inflammation in the brain. Therefore, we monitored associated activation of microglia to assess the possible role for inflammation in the cognitive outcomes (Meshi et al., 2006).

A key feature of the present study is that three behavioral tasks were first established to show benefits from exercise so that the manipulation of neurogenesis could be evaluated for a role in the performance *gains*. These three tasks were the Morris water maze test of spatial reference memory (van Praag et al., 1999b), rotarod test of motor performance (Pietropaolo et al., 2006), and contextual fear conditioning (Baruch et al., 2004). Our predictions were the following. Intact neurogenesis would *not* be required for baseline performance on the water maze. Several studies have established no change in baseline levels of water maze performance after neurogenesis is reduced using irradiation or chemical toxin methods (e.g. MAM) (Shors et al., 2002; Madsen et al., 2003; Raber et al., 2004; Snyder et al., 2005), although some have seen small decrements depending on methodology (Rola et al., 2004; Snyder et al., 2005; Zhang et al., 2008). Though we expected no change in baseline, we predicted reduced neurogenesis would eliminate the gain in performance from exercise. This is based on the assumption that new neurons provide new units that can be molded by experience (van Praag et al., 2005). Although rodents may only use a small fraction of granule cells during the water maze task (Kee et al., 2007), new neurons have been hypothesized to display greater plasticity than older neurons, and hence we predicted that availability of large numbers of new neurons would facilitate improved spatial learning and memory on this task. More specifically, we hypothesized that whereas older neurons may be sufficient for baseline performance on the water maze, improved performance would require the full complement of large numbers of new neurons recently incorporated into the network and available for plasticity.

We hypothesized that intact neurogenesis would *not* be required for enhanced performance from exercise on rotarod because rotarod is thought to rely principally on the cerebellum (Goddyn et al., 2006) which was spared from radiation under the lead shield (i.e. rotarod was included as a negative control). We were less certain about what to predict for contextual fear conditioning. The role of hippocampus in contextual fear conditioning has been debated (Cho et al., 1999; Gewirtz et al., 2000; Lopez-Fernandez et al., 2007). Recent studies suggested that new neurons are required for baseline levels of contextual fear conditioning in 129/SvEv mice (Saxe et al., 2006) and male Long Evans rats (Wojtowicz et al., 2008). On the other hand, in C57BL/6J, ibotenic acid lesions of the hippocampus do not prevent freezing to context (Gerlai, 2001). Hence, for C57BL/6J, we predicted older neurons and reduced neurogenesis would be sufficient for baseline performance, and that stress hormone signaling subsequently induced from fear (e.g. glucocorticoid, epinephrine or norepinephrine) in other parts of the brain and body might outweigh contributions of hippocampal neurogenesis for freezing behavior.

EXPERIMENTAL PROCEDURES

Animals

A total of 42 male and 42 female mice from the C57BL/6J standard inbred strain were studied. C57BL/6J was chosen because a strong correlation between exercise, increased hippocampal neurogenesis and enhanced learning and memory on the Morris water maze has been established for this strain (van Praag et al., 1999b, 2005).

The experiments were conducted in two batches that varied slightly with regard to the parameters as indicated. Both batches were combined for behavioral performance analysis (water maze, rotarod and fear conditioning) (n=42 males and 42 females). Only batch 1 was analyzed for hippocampal neurogenesis and microglia activation (n=20 males and 19 females). See Fig. 1A.

Husbandry

Animals arrived at the Beckman Institute Animal facility from The Jackson Laboratory at 5 weeks of age. Upon arrival they were housed four per cage by sex in standard polycarbonate shoebox cages with corncob bedding, 7097 ¼″ (Harlan Teklad, Madison, Wisconsin, USA) until they were individually housed either in standard shoebox cages (without filter tops) or cages with wheels as described below. Rooms were controlled for temperature (21±1 °C) and photo-period (12-h L:D; lights on at 7 am and off at 7 pm). Food (Harlan Teklad 7012) and water were provided *ad libitum*. The Beckman Institute Animal Facility is AAALAC approved. All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee and adhered to NIH guidelines. All efforts were made to minimize the number of animals used and their suffering.

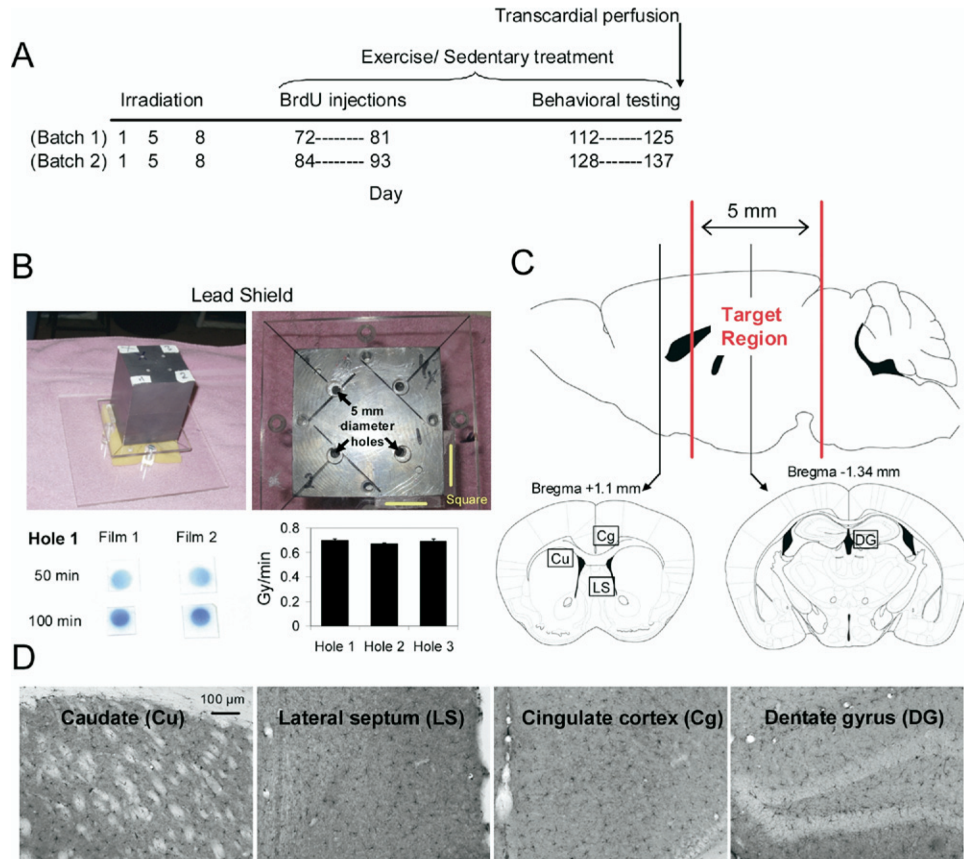


Fig. 1. Experimental design, irradiation methods, and detection of inflammation. (A) Schematic diagram of the experimental design. Relevant time-points are shown for each batch to illustrate the slight differences. (B) Photos of the lead shield used to direct gamma rays through the hippocampus of mice. Dimensions of the lead are 11.4×7.6×7.6 cm, holes are 5 mm diameter. Top right is the same lead shield shown on the left, flipped over, with the base removed, clear plastic top unscrewed, and squared off on top of the lead shield. Three mice were placed between the yellow foam pad and the clear plastic top (top left; top of the heads would be facing up, noses together). The heads of the mice were pressed into place and oriented such that the posterior base of the eyes were tangent to the horizontal lines and center of the head intersecting with the perpendicular lines (top right; top of the heads would be facing down). Note that the apparatus was designed for four animals but one of the holes was not drilled at the correct angle and hence did not transmit the same amount of radiation as the other holes and so could not be used. Bottom left panel shows an example of film placed under hole number 1 after different durations of gamma radiation exposure used as raw data for dosimetry. Bottom right shows the average estimate of Gy/min±S.E. for each hole based on 6 to 10 independent film samples. (C) Schematic diagram of the mouse brain shown first in sagittal plane to indicate target region for irradiation and then coronal planes showing locations where brain sections were photographed and analyzed for numbers and sizes of microglia (or macrophages) using immunohistochemical detection of cd68. (D) Examples of cd68 stain in each brain region sampled. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

Irradiation

After 15 days acclimation to the animal facility (batch 1) or 31 days (batch 2), mice (age 50 or 66 days) were split into two groups, Sham or Irradiated. A Cobalt-60 Theratron 780 unit at the University of Illinois Vet School was used. Source activity is 120.5 cGy/min. A lead shield was built to collimate gamma radiation to a 5 mm diameter beam that was directed through the head (from dorsal to ventral) in the region of the hippocampus, sparing all other body parts. This was accomplished by arranging the animals under a custom-built stereotaxic apparatus (see Fig. 1B). Irradiated mice (n=48) received three sessions of 5 Gy separated by 3- or 4-day intervals. In order to immobilize animals during irradiation, animals were anesthetized with an i.p. injection of 70 mg/kg sodium pentobarbital. At this time animals also received an ear punch (left, right, neither or both) for individual identification by cage. Sham mice were anesthetized and ear punched, but were not exposed to radiation.

After irradiation, mice remained undisturbed for 64 days (batch 1) or 76 days (batch 2) before entering the exercise (or sedentary) phase of the experiment. This recovery period was chosen because Meshi et al. (2006) observed that radiation-induced increases in number of cd68-positive cells in the brain (microglia or macrophages; marker of inflammation) were no longer apparent after 2 months.

Exercise vs. sedentary treatments

Mice (114 or 142 days old), either Irradiated or Sham (from above), were placed individually in cages either without (Sedentary) or with running wheels (Runners) for 54 days. The first 10 days all mice received daily injections of 50 mg/kg bromodeoxyuridine (BrdU) to label dividing cells. Note that mice were deliberately not housed in cages with locked wheels because mice climb in locked wheels (Koteja et al., 1999; Rhodes et al., 2000, 2003b) and we wanted to keep physical activity to a minimum in the sedentary group.

The sample sizes for the groups were as follows:

Batch 1: Sham Sedentary (n=4 males, 4 females), Sham Runner (n=4 males, 4 females), Irradiated Sedentary (n=6 males, 5 females), Irradiated Runner (n=6 males, 6 females).
Batch 2: Sham Sedentary (n=5 males, 6 females), Sham Runner (n=6 males, 5 females), Irradiated Sedentary (n=6 males, 6 females), Irradiated Runner (n=5 males, 6 females).

Dimensions of running wheel cages were 36×20×14 cm (L×W×H) with a 23 cm diameter wheel mounted in the cage top. Dimensions of cages without wheels were 29×19×13 cm (L×W×H). Wheel rotations were monitored continuously in 1 min increments throughout the experiments via magnetic switches interfaced to a computer.

Behavioral performance

After 40 days of being housed with or without wheels, mice (age 154 or 182 days) were tested on three behavioral tasks, Morris water maze, rotarod, and contextual fear conditioning, in that order. This order was chosen so that the most complex task would be completed naïve (i.e. uninfluenced by performance on the other tasks). Fear conditioning was last so that it could not transfer fear to the other tasks. We opted for serial testing rather than counterbalancing to reduce inter-individual variation that might occur from testing animals in a different order. Testing took place during the light phase of the light/dark cycle in a different room than where animals were housed, except rotarod which was done in the same room. Animals were returned to cages with or without wheels immediately after testing. Hence, runners had continuous access to running wheels throughout the behavioral testing period. In batch 1, behavioral tasks were conducted over a series of 14 days with 2 days off between tasks whereas in batch 2 they were done over 10 days without any breaks between days.

Morris water maze

Mice were trained on Morris water maze, two trials per day for 5 days. A trial lasted either 60 s or after the mouse reached the platform and remained on the platform for 10 s. If a mouse did not reach the platform in 60 s it was gently guided there by hand. Mice were placed back in their cage and allowed to rest for 30 s between trials. One hour after training on day 5, the platform was removed and mice were tested with a probe trial (60 s).

Dimensions and parameters followed Wahlsten et al. (2005). The maze consists of a circular tub, 70 cm diameter and 20 cm deep. A platform made of white plastic mesh 8.5 cm square was placed in the middle of one quadrant submerged 0.5 cm below the surface of the water. Sixty milliliters of Crayola white tempera paint was added to the water to make the water sufficiently opaque to hide the platform from sight. White was chosen to provide contrast for video tracking from above (black mouse on white background). Water temperature was maintained at 25–26 °C. Topscan (CleverSystems, Reston, VA,USA) video tracking software was used to measure path length, swim speed and duration spent in different quadrants of the maze.

Rotarod

After water maze, mice were tested for performance on a rotarod (AccuRotor Rota Rod Tall Unit, 63-cm fall height, 30 mm diameter rotating dowel; Accuscan, Columbus, OH, USA). Animals were placed on the dowel starting at 0 rpm. The dowel was then accelerated at 60 rpm/min. A photobeam at the base stopped the timer automatically when a mouse fell off the dowel. This was repeated four consecutive trials per day for 3 days.

Contextual fear conditioning

Following rotarod, mice were tested for contextual fear conditioning. Mice were divided into two groups equally by treatment, fear conditioned or control. All mice were placed into a fear conditioning chamber for 180 s on day 1 and day 2. On day 1, mice in the fear group received 2 foot-shocks (0.5 mA, duration 2 s) at 120 and 150 s. Mice in the control group did not receive any foot-shocks. On day 2, all mice were placed into the chamber for 180 s without any foot-shocks. The chamber consisted of a plastic cage (dim 32×28×30 cm L×W×H) with a wire grid bottom connected to a shock scrambler controlled by digital timer (Med Associates, St. Albans, VT, USA). The animal's movement was tracked using TopScan video tracking software. Freezing was measured as the total number of seconds when the animal's center of mass, as identified by TopScan, did not register horizontal movement (±1 mm).

Immunohistochemistry

Following behavioral testing, animals were anesthetized with 100 mg/kg sodium pentobarbital (i.p.) and then perfused transcardially with 4% paraformaldehyde in phosphate buffer solution (PBS; 0.287% sodium phosphate monobasic anhydrous, 1.102% sodium phosphate dibasic anhydrous, 0.9% sodium chloride in water). Brains were postfixed overnight, and then transferred to 30% sucrose in PBS. Brains were then sectioned using a cryostat into 40 μm thick coronal sections. Sections were placed into tissue cryoprotectant (30% ethylene glycol, 25% glycerin, 45% PBS) in 24 well plates and stored at −20 °C. Three separate one-in-six series of these sections (i.e. series of sections throughout the rostro-caudal extent of the brain with 240 μm increments separating each section, approximately nine sections) were stained in each of the following ways.

BrdU-DAB. Purpose: To detect BrdU-positive (newly divided) cells in the dentate gyrus. Free floating sections were washed in tissue buffering solution (TBS; 1.3% Trizma hydrochloride, 0.19% Trizma base, 0.9% sodium chloride) and then treated with 0.6% hydrogen peroxide in TBS for 30 min. To denature DNA, sections were treated for 120 min with a solution of 50% de-ionized formamide and 10% 20× SCC buffer, rinsed for 15 min in 10% 20× SCC buffer, then treated with 2 N hydrochloric acid for 30 min at 37 °C, then 0.1 M boric acid in TBS (pH 8.5) for 10 min at room temperature. Sections were then treated (blocked) with a solution of 0.3% Triton-X and 3% goat serum in TBS (TBS-X plus) for 30 min, and then incubated in primary antibody against BrdU made in rat (Accurate, Westbury, NY, USA) at a dilution of 1:100 in TBS-X plus for 72 h at 4 °C. Sections were then washed in TBS, treated with TBS-X plus for 30 min and then incubated in secondary antibody against rat made in goat at 1:250 in TBS-X plus for 100 min at room temperature. Sections were then treated using the ABC system (Vector, Burlingame, CA, USA) and stained using a diaminobenzidine kit (Sigma, St. Louis, MO, USA).

Triple-fluorescent label. Purpose: To determine the proportion of BrdU-positive cells in the dentate gyrus that differentiated into neurons, astroglia, or neither. The procedure above was repeated except for the following. A cocktail was used for the primary antibody step, rat anti-BrdU (1:50; Accurate, Westbury, NY, USA), mouse anti-neuronal nuclear protein (NeuN) (1:50; Chemicon, Billerica, MA, USA), and rabbit anti-S100β (1:200; Swant, Switzerland). Secondary antibodies made in goat were conjugated with fluorescent markers (Cy2-red, Cy3-green, Cy5-blue) at dilution of 1:200 and also delivered as a cocktail, Cy2 anti-rat, Cy3 anti-mouse, Cy5 anti-rabbit. ABC and diaminobenzidine steps were omitted.

cd68-DAB. Purpose: To detect microglia (or macrophages), as marker for inflammation, in irradiated versus sham-treated brains. Free floating sections were washed in PBS, then treated with 0.6% hydrogen peroxide in PBS for 30 min. Tissue was blocked with a solution containing 0.3% Triton X and 3% normal

goat serum (PBS-X plus) for 60 min, then incubated for 72 h at 4 °C with rat anti-cd68 (1:400; Serotec, Raleigh, NC, USA) made in rat (1:400 dilution in PBS-X plus). Sections were then washed in PBS, treated with PBS-X plus for 30 min and then incubated in secondary antibody against rat made in goat at 1:250 in PBS-X plus for 90 min at room temperature. Sections were stained using the ABC system (Vector) with diaminobenzidine as chromogen.

Image analysis

BrdU-DAB. The entire granule layer (bilateral), represented in the one-in-six series, was photographed by systematically advancing the field of view of the Zeiss brightfield light microscope, and taking multiple photographs, via axiocam interfaced to computer, under 10× (total 100×) magnification. A large depth of field was used so that all particles within the section were visible in each photograph. These photographs were then analyzed using ImageJ software. In each image, the granule layer was traced, and BrdU-positive nuclei were counted within the traced region automatically by setting a fixed threshold to remove the background. In addition the area (pixels) within the trace was recorded. Number of BrdU positive cells were also counted by hand in four sections from each group ($n=20$ total) and values were regressed against the automated numbers. The R^2 value was 0.95 and the equation was $y=1.2\ (X)+1.07$, indicating that the automated method slightly underestimated numbers of cells as numbers increased due to the program being unable to distinguish multiple cells if they were touching each other. Hence, values were corrected using this equation. These data were used to generate estimates of total number of BrdU positive cells per cubic micrometer dentate gyrus sampled. Values were further adjusted by removing the fraction of cells predicted to cross the boundary of the section on one side to produce stereological, unbiased estimates. Results of all the statistical tests (below) were conducted using these adjusted (unbiased) values and un-adjusted, raw BrdU counts (biased) values, for comparison. Results were the same. Only results for the adjusted, unbiased values, are shown.

Triple label. A total of 1710 dentate gyrus BrdU-positive cells from 34 mice were micro-analyzed by confocal microscopy to identify whether each cell co-expressed NeuN, S100β or neither. Number of new neurons per cubic micrometer per mouse was calculated as number of BrdU cells per cubic micrometer (from above) multiplied by average proportion of BrdU cells co-expressing NeuN for designated group (e.g. sham sedentary, sham runner, irradiated sedentary, irradiated runner).

cd68-DAB. Numbers and sizes of microglia were measured in four different brain regions around the targeted area, the cingulate cortex, lateral septum, caudate, and dentate gyrus. See Fig. 1C. The first three regions were sampled approximately 1 mm anterior to the target region. The dentate gyrus was the target. Four photographs were taken of each region (both hemispheres in two adjacent sections nearest the coordinates shown in Fig. 1C) under 10× magnification (Zeiss brightfield light microscope) using a large depth of field so all particles within sections were visible. For cingulate cortex, lateral septum and caudate, all cd68-positive cells in the photo were counted automatically and measured for average size in pixels by setting a threshold to remove background in ImageJ. For dentate gyrus, the granule layer was traced, and only cd68-positive cells within the traced region were counted. Numbers of cells per volume were calculated and then adjusted by removing the fraction of cells predicted to cross the boundary of the section on one side to produce stereological, unbiased estimates.

Statistical analysis

Data were analyzed using SAS or R statistical software. In all analyses, $P<0.05$ was considered statistically significant. The following variables were analyzed using a two-way ANOVA with exercise (runner vs. sedentary) and irradiation (irradiated vs. sham) as the two factors: number of new neurons in the dentate gyrus per cubic mm, volume of the dentate gyrus (cubic mm), number of cd68-positive cells in the cingulate cortex, lateral septum, caudate, and dentate gyrus per cubic mm, size of cd68-positive cells (cubic micron), duration (s) in the target quadrant of the water maze during the probe trial, duration (s) freezing on test day of fear conditioning. These data were also analyzed including sex as a factor. In these linear multiple regression analyses, interactions between sex and the other factors were also tested for statistical significance.

The following data were analyzed by repeated measures analysis with day as the within subjects factor and treatment (four levels, sham-sedentary, sham-runner, irradiated-sedentary, irradiated runner) as the between subjects factor: latency (s), path length (m) and swim speed (m/s) to the hidden platform, and latency to fall off the rotarod (s) over days. These data were also analyzed by multiple linear regression including sex and interactions between sex and the other factors in the model.

Proportion of BrdU cells differentiating into neurons, glia or neither was analyzed by logistic regression, where proportion (binomial response) was modeled as a linear function of factors exercise, irradiation and their interaction. These proportion data were also analyzed including sex and interactions between sex and the other factors in the logistic regression.

RESULTS

Irradiation

See Fig. 1B. Gafchromic film MD-55, calibrated at the National Institute of Standards and Technology, was placed under each hole and around the apparatus for varying durations of radiation exposure. The shield, positioned directly under a 4×4 cm square beam emitted from the source, exposed approximately 0.7 Gy/min through the 5 mm holes. This was true for only three of four holes because one hole was accidentally not drilled at the correct angle of 1.65° off the central axis during machining. Negligible radiation was detected around the edges of the shield.

All animals survived radiation (three sessions of 5 Gy under the lead shield) and appeared to fully recover after anesthesia. After a few weeks, some animals developed visible changes in coat color on the top of the head (in the shape of a circle where the head was exposed to radiation).

Microglia

See Table 1 and Fig. 1D. Exactly 111 days following radiation, when animals were killed (age 168 days), numbers and sizes of cd68-positive cells were similar across all groups in all regions, with one exception. Size of cd68-positive cells in the dentate gyrus was slightly larger ($P<0.05$) in irradiated as compared with sham.

Wheel running

See Fig. 2. Radiation did not affect wheel running. Average distance traveled over the 54-day period was 5.7 km/day (±0.36 S.E.) for irradiated mice and 5.8 km/day (±0.42) for sham mice. Females ran 17% further than males ($P<0.05$).

Table 1. Mean (S.E.) number of microglia (cd68-positive cells), per cubic mm and size of microglia (cubic micron) in four different brain regions by treatment groups and associated two-way ANOVA statistics

Brain region	Sham		Irradiated		Two-way ANOVA F statistics and P-values		
	Sedentary	Runner	Sedentary	Runner	Radiation	Exercise	Interaction
Number microglia per mm^3 ($\times10^3$)							
Cingulate cortex	13.3 (1.54)	10.8 (1.00)	12.1 (1.60)	14.9 (2.37)	$F_{1,32}$=0.63, P=0.43	$F_{1,32}$=0.05, P=0.83	$F_{1,32}$=2.14, P=0.15
Lateral septum	15.0 (3.10)	8.9 (1.53)	10.5 (1.71)	12.1 (1.26)	$F_{1,33}$=0.10, P=0.75	$F_{1,33}$=0.86, P=0.36	$F_{1,33}$=3.92, P=0.06
Caudate	10.3 (2.62)	7.3 (1.24)	7.3 (0.96)	9.4 (1.57)	$F_{1,35}$=0.05, P=0.82	$F_{1,35}$=0.00, P=0.99	$F_{1,35}$=2.50, P=0.12
Dentate gyrus	14.7 (1.91)	12.3 (1.55)	16.1 (2.11)	14.5 (1.40)	$F_{1,35}$=0.89, P=0.35	$F_{1,35}$=1.22, P=0.28	$F_{1,35}$=0.04, P=0.84
Size of microglia (μm^3)							
Cingulate cortex	103 (8.4)	102 (10.2)	104 (8.0)	116 (10.3)	$F_{1,32}$=0.58, P=0.45	$F_{1,32}$=0.43, P=0.52	$F_{1,32}$=0.47, P=0.50
Lateral septum	121 (6.9)	102 (12.1)	114 (8.4)	125 (5.5)	$F_{1,33}$=0.93, P=0.34	$F_{1,33}$=0.04, P=0.84	$F_{1,33}$=3.40, P=0.07
Caudate	79 (9.6)	74 (9.7)	86 (5.1)	99 (8.5)	$F_{1,35}$=3.85, P=0.06	$F_{1,35}$=0.50, P=0.49	$F_{1,35}$=1.33, P=0.26
Dentate gyrus	91 (5.5)	90 (6.7)	105 (6.3)	104 (5.8)	$F_{1,35}$=5.09, P=0.03	$F_{1,35}$=0.02, P=0.88	$F_{1,35}$=0.00, P=0.95

Neurogenesis

See Fig. 3. Running increased neurogenesis approximately fourfold ($P<0.001$) whereas 15 Gy reduced neurogenesis by approximately 50% ($P<0.001$). A larger reduction in absolute numbers of new neurons occurred in runners as compared with sedentary animals (interaction, $P<0.05$).

These changes in neurogenesis were attributed primarily to number of BrdU positive cells in the granular layer (Fig. 3A). However, radiation also slightly reduced the proportion of BrdU cells differentiated as neurons ($P<0.001$) and increased proportion of undifferentiated cells ($P<0.01$), whereas exercise did the reverse. Exercise increased proportion of neurons ($P<0.01$) and decreased proportion undifferentiated ($P<0.05$) (Fig. 3B). Radiation also slightly reduced proportion of new cells differentiated as glia ($P<0.05$).

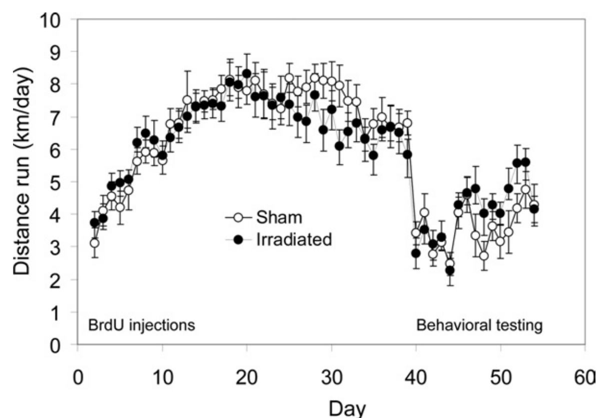


Fig. 2. Wheel running over the course of the study. Distance run (km/day) (±S.E.) for batch 1, shown separately for irradiated and sham mice. The first 10 days mice received daily injections of 50 mg/kg BrdU to label dividing cells. The last 14 days mice were tested on three behavioral tasks, during the light phase of the light dark cycle when levels of wheel running are negligible. Data for batch 2 (not shown) are nearly identical. Increased wheel running over the first 20 days is typical for mice. The decrease at day 40 reflects delayed effects of behavioral testing on activity during the dark phase, not reduced amount of time spent in the cage with a wheel. Animals were removed from wheels for only brief periods for behavioral testing at times when levels of running are negligible.

Changes in neurogenesis in the groups were associated with corresponding, though smaller, changes in volume of the dentate gyrus. Among all individuals, number of new neurons was correlated with volume of the dentate gyrus (R^2=0.26; $P<0.001$). Running increased volume by approximately 10% ($P<0.001$) whereas irradiation reduced volume by approximately 7% ($P<0.01$). These effects were additive (no statistical interaction) (Fig. 3C).

Interesting sex differences were also observed. Across all groups, on average, males displayed 75% the number of new neurons per volume dentate gyrus as females ($P<0.01$), and irradiation produced a larger reduction in number of new neurons in females than males (interaction between sex and irradiation treatment was significant, $P<0.05$). In the irradiated group, males showed slightly reduced proportion BrdU cells differentiated into neurons as compared with females (78%±2.2 S.E. versus 87%±1.8), but in the sham group proportions were identical between sexes (89%±1.5 S.E. versus 89%±1.3). This was reflected by a main effect of sex ($P<0.05$), and significant interaction between sex and irradiation treatment ($P<0.05$).

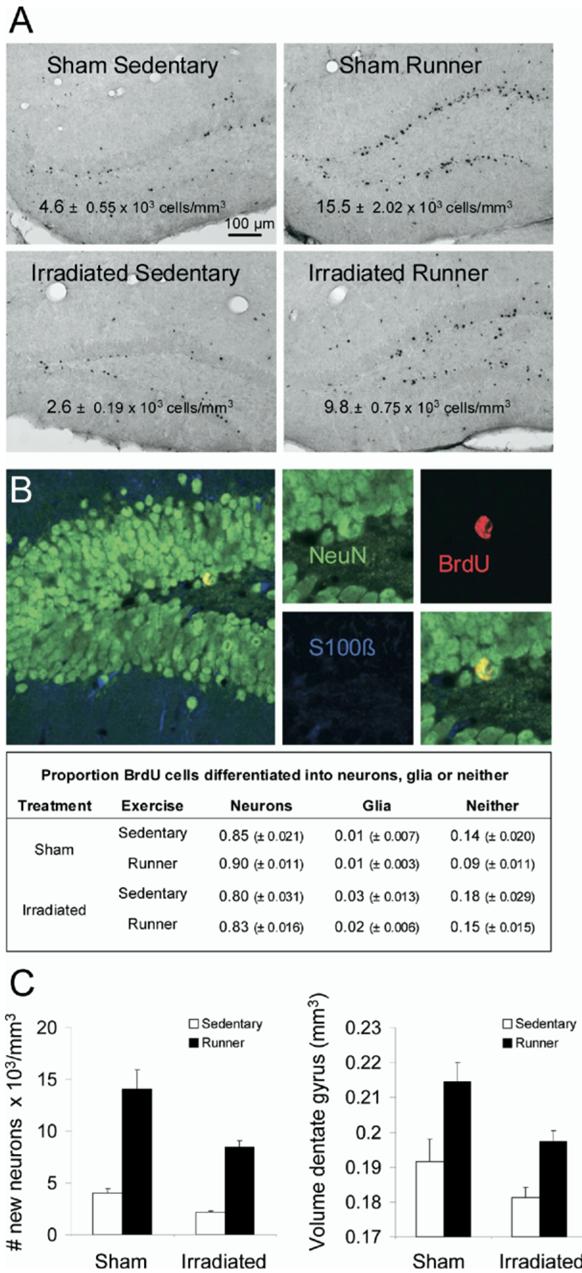
Behavioral performance

Morris water maze; see Fig. 4. Acquisition (Fig. 4A): All animals learned the Morris water maze as indicated by decreased latency or path length with day (all $P<0.001$). The learning curves were steeper than has been reported for larger mazes (van Praag et al., 1999b; Rhodes et al., 2003b). The interaction between exercise treatment and irradiation was marginally non-significant for path length (P=0.09). In sham mice, exercise improved learning as measured by decreased path length (main effect, $P<0.05$), but exercise had no effect in irradiated mice. In both batches, sham runners showed the steepest average learning curves as compared with the other groups for path length and latency, though effects for latency were smaller and not statistically significant. Swim speed significantly decreased as the days passed from an average of 143.5–

119.6 mm/s±3.2 ($P<0.001$) but did not differ between treatment groups.

Probe test (Fig. 4B): Each group, when analyzed separately, displayed significantly more time in the target quadrant as compared with any other quadrant during the probe test on day 5 ($P<0.001$ for each group). Running significantly enhanced time spent in the target quadrant and number of platform crossings in sham mice but not in irradiated mice relative to the sedentary groups. This was indicated by significant interaction between irradiation and exercise treatment ($P<0.05$). These results were consistent in both batches. No main effects of irradiation were detected.

Sex differences were as follows (data not shown). During acquisition, males displayed shorter path length to the platform on day 1 (6.2 versus 8.0 m±0.25 S.E.), and similar length by day 5 (both 1.4 m±0.25 S.E.). This resulted in significant main effect of sex ($P<0.001$) and interaction between sex and day for path length ($P<0.001$). Females swam slightly faster than males



Proportion BrdU cells differentiated into neurons, glia or neither

Treatment	Exercise	Neurons	Glia	Neither
Sham	Sedentary	0.85 (± 0.021)	0.01 (± 0.007)	0.14 (± 0.020)
	Runner	0.90 (± 0.011)	0.01 (± 0.003)	0.09 (± 0.011)
Irradiated	Sedentary	0.80 (± 0.031)	0.03 (± 0.013)	0.18 (± 0.029)
	Runner	0.83 (± 0.016)	0.02 (± 0.006)	0.15 (± 0.015)

Fig. 3. Adult hippocampal neurogenesis. (A) Photographs of the dentate gyrus stained for BrdU, showing representatives from each of the four groups. Black dots are nuclei stained positive for BrdU (indicating newly divided cell). Also reported within each picture is the average number of BrdU-positive cells (±standard error) per volume dentate gyrus for each group. (B) Photographs of the dentate gyrus of a sham sedentary mouse triple stained, green for NeuN (mature neuronal marker), red for BrdU, and blue for S100β (astroglia marker). Panels to the right show the tissue illuminated for each color separately and combined zoomed in around the BrdU cell indicating an episode of neurogenesis. The table shows the proportion of BrdU cells (±S.E.) differentiated into neurons (NeuN), glia (S100β) or neither. (C) Average number of new neurons per volume dentate gyrus and estimated average volume shown separately by group. Note that in the volume graph, the *y* axis starts at 0.17 cubic mm to facilitate comparison of relatively small differences between groups. Standard error bars shown.

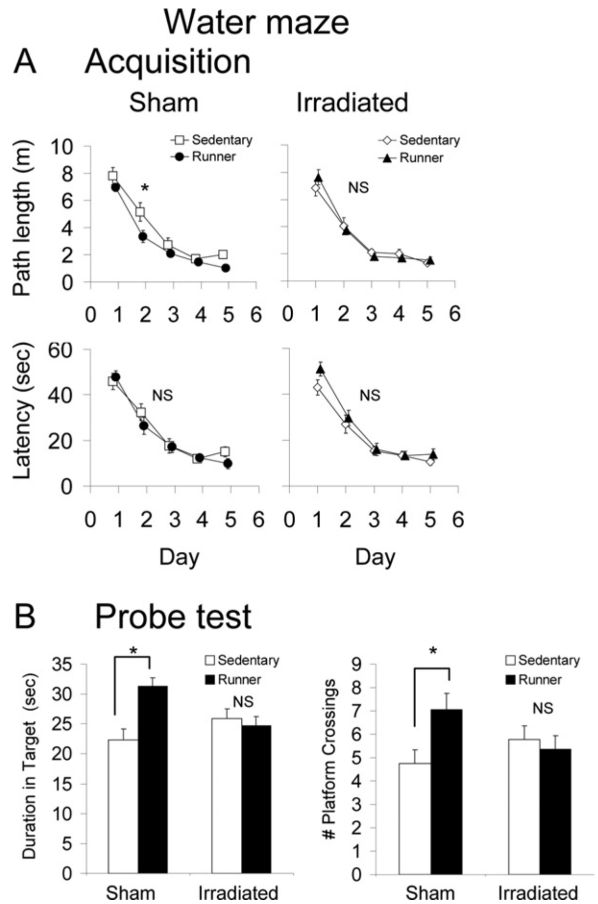


Fig. 4. Morris water maze. (A) Acquisition of the maze shown separately for sham (left panel) versus irradiated (right panel). Within each graph, runners are shown as filled symbols and sedentary as open symbols. Changes in path length (m) and latency (s) across the days are shown. The star in the top left panel indicates significant main effect of exercise ($P<0.05$). (B) Probe test results shown as duration in the target quadrant (s) and number of crossings through the platform location. Note that running enhanced performance in sham animals as indicated by the stars ($P<0.05$), but not in irradiated animals.

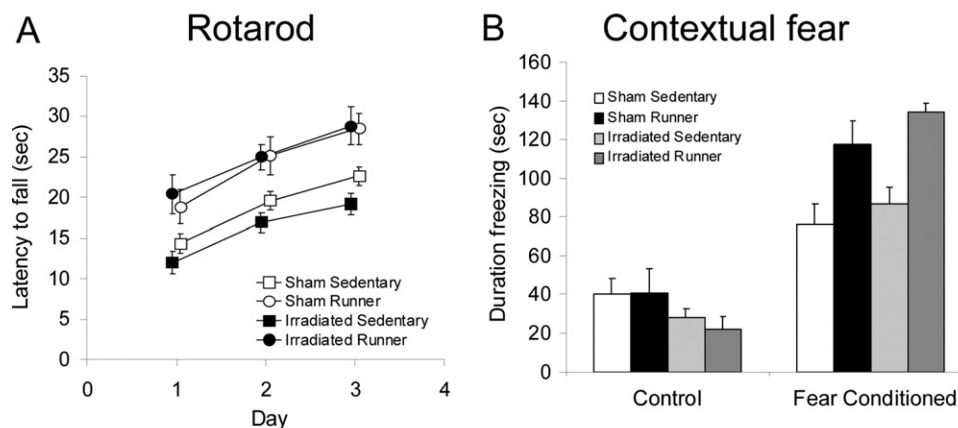


Fig. 5. Rotarod and contextual fear. (A) Latency (s) to fall off the accelerating rotarod over 3 days (each day the average of four trials), shown by group. (B) Duration of freezing (s) on day 2 for animals that did *not* receive shocks in the chamber on day 1 (Control) versus those that received shocks in the chamber on day 1 (Fear conditioned), shown separately by treatment group. Standard error bars shown.

(13.7 cm/s versus 12.5 cm/s±0.34 S.E.; $P<0.01$) which resulted in no significant sex effects for latency to the platform, though a marginally non-significant interaction was observed between sex and day ($P=0.10$) with females starting off on day 1 at an average of 50 s and ending on day 5 with 11.8 s, whereas males started at 45 and ended at 13.5 s (±1.7 S.E.). These effects occurred across treatment groups (i.e. interactions between sex and irradiation or exercise, were not significant). No sex differences were observed on the probe test.

Rotarod; see Fig. 5A. All animals learned the rotarod as indicated by increased latency to fall with day ($P<0.001$). Learning curves did not differ between groups (i.e. interaction between day and treatment group was not significant). However, runners displayed elevated performance above sedentary on day 1 and this difference was maintained in parallel as all groups learned the task ($P<0.001$). Irradiation had no effect. An interesting sex difference was observed with females performing slightly better than males across groups (latency to fall was 17% higher in females than males; $P<0.05$).

Fear conditioning; see Fig. 5B. Running increased duration of freezing on day 2 (the test day) in animals where context was paired with foot-shock ($P<0.001$). Irradiation had no effect. No group differences occurred for animals in which context was *not* paired with shock. No sex effects were detected.

DISCUSSION

As compared with other vertebrates and invertebrates, mammals have restricted ability to generate new nerve cells in adult life (Lindsey and Tropepe, 2006). In only two regions of the adult mammalian brain is there undisputed evidence for substantial neurogenesis in adulthood: hippocampus and olfactory bulb (Altman and Das, 1965, 1966; Gould, 2007). Despite great progress unraveling the cellular and molecular biology and behavioral relevance associated with adult mammalian hippocampal neurogenesis, the functional significance, if any, for the phenomenon has remained a mystery (Kempermann et al., 2004; Lindsey and Tropepe, 2006). Recent data collected within the last 10 years has established that adult hippocampal neurogenesis is regulated by a variety of factors including stress (Gould et al., 1991), environmental enrichment (Kempermann et al., 2002), exercise (van Praag et al., 1999a), antidepressant treatment (Malberg et al., 2000; Santarelli et al., 2003), genetics (Kempermann et al., 2006), dietary restriction (Lee et al., 2000), alcohol intake (Herrera et al., 2003), among others.

One of the most potent factors known to increase adult hippocampal neurogenesis is aerobic exercise (Rhodes et al., 2003b). Exercise training produces a massive increase in new neurons in mice, e.g. fourfold in this study, four- to fivefold in Rhodes et al. (2003b). Researchers have speculated that exercise-induced neurogenesis might contribute to enhanced cognitive performance on spatial tasks (Rhodes et al., 2003b; van Praag et al., 2005; Kramer et al., 2006; Trejo et al., 2008). The present study adds to this literature by confirming that intact neurogenesis is required for enhanced spatial memory from exercise in C57BL/6J mice. These results have broad implications for aging, stress, trauma, stroke, or neurodegenerative disease because they suggest it is possible to improve cognition by stimulating growth of new nervous tissue in the brain.

An important feature of this study is that we first established gains in performance (from exercise) on three behavioral tasks with varying sensitivity and dependence on function of the hippocampus, and then asked whether or not these gains were abolished in animals that exercise at equivalent levels (Fig. 2) but with reduced neurogenesis (Fig. 3). As predicted, baseline performance on the tasks was not sensitive to irradiation (Shors et al., 2002; Madsen et al., 2003; Raber et al., 2004; Snyder et al., 2005). This was a useful result because if baseline performance was affected, changes due to exercise would be more difficult to interpret.

The observation that baseline performance on the water maze can be accomplished with reduced neurogenesis but that improved performance from exercise requires in-

tact neurogenesis suggests a specific contribution of new neurons to cognitive *gain*. Moreover, results suggest that only the full complement of new neurons, or at least more than 50%, is required, possibly by reaching a threshold for availability of highly plastic units that can be molded by experience. Note that a mere increase in neurogenesis was not sufficient to enhance performance (i.e. the total appears to be what matters), because the irradiated group showed increased neurogenesis without cognitive gain.

As predicted, intact neurogenesis was not required for gains in performance on the rotarod (Fig. 5A) or contextual fear (Fig. 5B). The rotarod data are consistent with the idea that motor performance is not strongly dependent on hippocampus (Goddyn et al., 2006) but the implication for contextual fear is more complex (Fig. 5B). In C57BL/6J, ibotenic acid and electrolytic lesion studies show that the acquisition and display of contextual fear is not dependent on hippocampus (Frankland et al., 1998; Gerlai, 2001). Moreover, neurogenesis was not required for baseline expression of contextual fear in transgenic mice backcrossed onto C57BL/6J (Zhang et al., 2008). However, this result does not appear to translate to other genotypes and species where recent studies indicate that intact adult hippocampal neurogenesis is required for baseline display of contextual fear (using similar methodology for the behavior) in 129/SvEv mice (Saxe et al., 2006) and male Long Evans rats (Wojtowicz et al., 2008).

To our knowledge this is the first study to manipulate hippocampal neurogenesis to examine the role in enhanced behavioral performance from exercise. A recent study by Wojtowicz et al. (2008) examined the effects of running and of inhibiting adult neurogenesis on learning and memory male Long Evans rats, but the rats did not show gains in behavioral performance from exercise on either contextual fear or water maze, so the data are not relevant for evaluating the hypothesis that performance gains require intact neurogenesis. Meshi et al. (2006) recently used the irradiation strategy to evaluate the role of neurogenesis in enhanced learning on water maze in response to "environmental enrichment" in 129Sv/Ev mice. In that study mice were housed either in standard cages (four per cage) or in "enriched" cages (eight per cage) which were larger and included running wheels, toys, and nesting material. Neurogenesis was reduced to trace levels using focal x-irradiation of the hippocampus but irradiated mice displayed the same profile of enhanced learning and memory on the water maze in response to enrichment as non-irradiated mice.

Similar data were observed in Fan et al. (2007) for Mongolian gerbils, where x-irradiation did not eliminate improvement in water maze performance from environmental enrichment despite an approximate 70% reduction in neurogenesis. Inconsistent results may be due to genotype or species, because correlations between exercise, spatial learning and neurogenesis are strong and well established in C57BL/6J not 129Sv/Ev mice or Mongolian gerbils (van Praag et al., 1999b, 2005). Previous data for outbred versus selectively bred lines of Hsd:ICR mice (Rhodes et al., 2003b), and unpublished data in our laboratory comparing C57BL/6J with DBA/2J, demonstrate that genotypes vary dramatically for exercise-induced neurogenesis and pro-cognitive responses to exercise. Another possibility is that in Meshi et al. (2006) and Fan et al. (2007), the other enrichment factors (e.g. larger cages and social groups) might have improved spatial memory via mechanisms independent of neurogenesis. Further, it is possible that levels of wheel running were lower in 129Sv/Ev than C57BL/6J mice (Lightfoot et al., 2004). Levels of running were not reported in Meshi et al. (2006). However, a previous study found that average levels of running in 129Sv/Ev mice ranged from 24 m per day to 4.7 km per day (Allen et al., 2001), whereas C57BL/6J run between 5 and 10 km/day (Fig. 2).

In addition to relevance for regenerative medicine, results also have important clinical implications for patients receiving cranial radiation treatment for brain tumors. Reduced neurogenesis is associated with cognitive decline in these patients (Monje and Palmer, 2003; Raber et al., 2004). The present results suggest that exercise could be used as a tool to increase neurogenesis. This is consistent with Fan et al. (2007) and Wojtowicz et al. (2008), but not Meshi et al. (2006) where no recovery in neurogenesis after irradiation was observed from environmental enrichment. On the other hand, this study shows that irradiated runners still had 50% fewer new neurons than sham runners and this difference seems to be important since exercise did not improve spatial memory in irradiated runners as it did in sham runners (Fig. 4).

Methodological considerations

We interpreted lack of improvement in performance on the water maze as a consequence of reduced neurogenesis. An alternative explanation is that side effects of irradiation, other than reduced neurogenesis (e.g. inflammation, cell death, impaired synapses or dendrites), caused the deficits. Note that inflammation itself can reduce neurogenesis (Monje et al., 2003), making these variables difficult to separate. Nonetheless, several pieces of evidence argue against this alternative. First, the vast majority of neurons in the mammalian CNS are no longer dividing (Rakic, 2002) and hence are least sensitive to radiation injury (Peissner et al., 1999). This is consistent with results of the present study in which few side effects of radiation were observed. For example, irradiation had no effect on wheel running behavior (Fig. 2) and did not interfere with baseline performance on any of the behavioral tasks. Also, at the time of behavioral testing, minimal differences in inflammation (as measured by immunohistochemical detection of cd68) were observed in brain areas targeted for irradiation (see Table 1). This was expected based on previous work showing that inflammation subsides after approximately 2 months (Meshi et al., 2006). Results in Table 1 extend Meshi et al. (2006) by showing that some residual effects of inflammation are still detectable in the dentate gyrus as enlarged microglia (or macrophages). Taken together, measured side effects were minimal, but the possible influence of residual inflammation or other features

not measured operating independent of neurogenesis cannot be ruled out.

Consistent with previous studies, the gain from exercise on acquisition of the water maze in sham animals was small (Fig. 4A) (van Praag et al., 1999b, 2005; Rhodes et al., 2003b) and we cannot be certain that this difference reflects spatial learning as opposed to non-spatial strategies such as swimming back and forth in a systematic fashion or swimming in a circle the right distance from the edge of the maze. A visible platform test is sometimes used as a control to help tease apart these factors (Gerlai, 2001) but different strategies would likely be used in the visible case (e.g. swim toward the flag) diminishing value of these data. Moreover, previous studies have already established that visible platform learning on the water maze is not changed by exercise in mice (Rhodes et al., 2003b). In this study, the probe test data stand alone as valuable evidence that exercise enhanced the spatial contribution to solving the water maze in sham mice but not in irradiated mice (Fig. 4B) (van Praag et al., 1999b, 2005; Wahlsten et al., 2005).

Implications and future directions

Results suggest intact exercise-induced adult hippocampal neurogenesis is required for specific gains in cognitive performance. This conclusion has broad implications for aging, stress, trauma, stroke, or neurodegenerative disease because it suggests it is possible to improve cognition by stimulating growth of new nervous tissue in the brain. Therefore, effort toward understanding the microenvironment created by exercise responsible for stimulating neurogenesis has promise for regenerative medicine. We suggest it may be useful to take a systems approach here (e.g. genomic, proteomic) (Huang and Wikswo, 2007) because the microenvironment is likely going to require a large collection of changes in many molecules such as BDNF, IGF-1, but also possible changes in blood flow, blood vessel growth, and/or neural activity associated with exercise (Rhodes et al., 2003a; Ding et al., 2006; Holschneider et al., 2007; Trejo et al., 2008). An alternative approach is to identify genotypes that display strong versus weak gains from exercise to identify key genes underlying the development of a nervous system with high regenerative capacity (following the general strategy of Rhodes et al., 2007). The goal would be to identify molecular targets (or switches), capable of manipulating the entire system by adjusting a few components.

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