EVALUATING THE REGULATION AND FUNCTION OF ADULT MOUSE HIPPOCAMPAL NEUROGENESIS INDUCED FROM RUNNING

BY

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DISSERTATION

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ABSTRACT

Exercise is a potent natural stimulator of adult mammalian hippocampal neurogenesis. Relatively little is known about how exercise stimulates neuron formation in the hippocampus, and even less is known about the possible functions of new neurons. Therefore, factors regulating the survival and the potential functions of new hippocampal granule neurons generated from running were studied in adult mice. Chapter 1 examined the formation and activation of adult mouse granule cells over the course of access to running wheels. Granule cell activity (measured by c-Fos immunoreactivity) and net neurogenesis remained elevated at a relatively constant level over 50 days of running, despite observing a gradual increase in running distance over the first 20 days of wheel access. These results in conjunction with recent literature suggest that granule cell activity may be involved in regulating the stable, but elevated levels of neurogenesis, relative to animals housed without wheels, over the course of running. Chapter 2 investigated whether vasculature formation in the granule cell layer adapts to support the elevated level of granule cell activation and the constant addition of the new neurons over several days of running in adult mice. Results showed that vasculature density increased in the granule cell layer, but not across the entire hippocampus of chronically running mice. These data suggest that increased blood vessel density from running is specific to the granule cell layer within the hippocampus. An additional aim of chapter 2 was to test the hypothesis that new neurons from running are recruited into neural activity (measured by c-Fos immunoreactivity) displayed in the granule cell layer from running. The results showed that new neurons preferentially display c-Fos over mostly pre-existing neurons during running, suggesting that one potential function of new neurons is to process information about running behavior itself. Chapter 3 examined whether any form of physical activity can regulate neurogenesis and new and pre-existing granule cell activity (as measured by c-Fos, Zif268, and Arc immunoreactivity), or whether only more robust activity (such as running) is necessary these changes. Results showed that the expression of c-Fos, Arc, and Zif268 in new & pre-existing neurons, as well as net neurogenesis was strongly correlated distance traveled on running wheels, but not distance traveled in cages without running wheels. These data suggest that robust repetitive movements, such as
running, are necessary to stimulate neuronal activity and neurogenesis. Evidence from the previous chapters favor the hypothesis that a subset of neurons formed from running may function in processing information related to running behavior itself. The remaining chapters explore an alternative hypothesis that new neurons are highly plastic units that may function in whatever information the hippocampus is processing at any particular moment. Chapter 4 entertained the hypothesis that new neurons generated from running also contribute to improved performance on water maze, contextual fear conditioning, and rotarod. Groups of running and non-running mice were either irradiated to reduce neurogenesis or not irradiated. Results showed that running enhanced performance over sedentary mice on contextual fear conditioning and rotarod, regardless of irradiation. However, only non-irradiated running mice displayed improved performance on the water maze over irradiated runners, irradiated sedentary, and non-irradiated sedentary mice. These data suggest new neurons generated from running may increase plasticity that can be used to improve hippocampal-dependent spatial learning. Chapter 5 examines whether new neurons generated from running become activated in association with spatial learning tasks. The activation of new neurons (as measured by Zif268 immunoreactivity) was assessed in running and sedentary mice following participation in one of three different tasks that engages the hippocampus; the water maze, novel environment exploration, or wheel running. Results show that the proportion of new neurons displaying Zif268 was related to the degree of Zif268 induction in the granule cell layer from each task. Sedentary and runner mice did not differ in the proportion of new neurons expressing Zif268 within each task, demonstrating that a greater number of new neurons are becoming activated in running animals. Taken together, results favor the hypothesis that new neurons from running are highly plastic units that can become activated by distinct tasks that engage the dentate gyrus.
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CHAPTER 1

Adult hippocampal neurogenesis and c-Fos induction during escalation of voluntary wheel running in C57BL/6J mice.

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Abstract

Voluntary wheel running activates dentate gyrus granule neurons and increases adult hippocampal neurogenesis. Average daily running distance typically increases over a period of 3 weeks in rodents. Whether neurogenesis and cell activation are greater at the peak of running as compared to the initial escalation period is not known. Therefore, adult C57BL/6J male mice received 5 days of BrdU injections, at the same age, to label dividing cells during the onset of wheel access or after 21 days during peak levels of running or in sedentary conditions. Mice were sampled either 24 hours or 25 days after the last BrdU injection to measure cell proliferation and survival, respectively. Immunohistochemistry was performed on brain sections to identify the numbers of proliferating BrdU labeled cells, and new neurons (BrdU/NeuN co-labeled) in the dentate gyrus. Ki67 was used as an additional mitotic marker. The induction of c-Fos was used to identify neurons activated from running. Mice ran approximately half as far during the first 5 days as compared to after 21 days. Running increased Ki67 cells at the onset but after 21 days levels were similar to sedentary. Numbers of BrdU cells were similar in all groups 24 hours after the final injection. However, after 25 days, running approximately doubled the survival of new neurons born either at the onset or peak of running. These changes co-varied with c-Fos expression. We conclude that sustained running maintains a stable rate of neurogenesis above sedentary via activity-dependent increases in differentiation and survival, not proliferation, of progenitor cells in the C57BL/6J model.
**Introduction**

Before the 1990s it was widely believed that the adult mammalian brain could not generate new nerve cells but now it is established that adult neurogenesis occurs in the olfactory bulb and dentate gyrus in rodents (Cameron et al., 1993, Corotto et al., 1994), humans (Eriksson et al., 1998, Bedard and Parent, 2004), and non-human primates (Gould et al., 1999b). This discovery has generated great interest and enthusiasm because if we can understand how neurons regenerate and incorporate into networks in the adult brain, that could have broad applications for treatment of neurodegenerative disease, cognitive decline with aging, stroke, and possibly depression and anxiety.

Many environmental and genetic factors are associated with differential regulation of adult hippocampal neurogenesis (e.g., Lucassen et al. 2010, , Fuchs and Gould, 2000, Kempermann et al., 2006). One potent environmental factor that increases neurogenesis is aerobic exercise (van Praag et al., 1999b). Most studies that measured effects of exercise on neurogenesis in mice labeled cells born at the onset of access to running wheels. These studies have demonstrated that running enhances cell proliferation, differentiation, and the survival of new neurons (van Praag et al., 1999b, Brown et al., 2003, Rhodes et al., 2003b, Clark et al., 2008, Clark et al., 2009). By influencing these factors, voluntary running can lead to as much as a 4-fold increase in the number of new neurons that become integrated into the granule cell layer (Rhodes et al., 2003b, Clark et al., 2009).

However, recent studies suggest that the regulation of adult hippocampal neurogenesis by running changes over the course of exercise training. For example, cell proliferation returns to sedentary levels after approximately 19 days of running in C57BL/6 (Kronenberg et al., 2006, Fuss et al., 2009, Snyder et al., 2009b). Presently, it is not known to what extent changes in neurogenesis over the course of exercise training are related to the escalation of running.

It is well established in mice and rats that average daily running distance increases during the initial days of running and then reaches a plateau after several days (Momken et al., 2004, Fuss et al., 2009, Klaus et al., 2009, Leasure and Decker, 2009, Van der Borght et al., 2009). In C57BL/6J male mice, we reliably find levels of running reach a
peak at approximately day 20 and thereafter maintain a plateau (Clark et al., 2008, Clark et al., 2009). To the best of our knowledge, no one has directly examined whether neurogenesis (the net result of proliferation, differentiation and survival) is greater during the later stages of voluntary running, when running distance is greater. Although Kronenberg et al. (Kronenberg et al., 2006) examined proliferation and survival of new neurons at three different time-points over a 32 day running period, the mice did not escalate their running over the days for unknown reasons. The commonly displayed natural increase in wheel running distance creates a useful model to explore how voluntary increases in running over time affects the formation of new neurons in the hippocampus.

One feature of dentate gyrus granule neurons that is not widely known or appreciated, is that they are acutely and quantitatively activated (as measured by c-Fos expression or electrical recording) from running, with faster running speeds associated with proportionally greater activation (Oladehin and Waters, 2001, Rhodes et al., 2003a, Bland et al., 2006, Clark et al., 2009). Although it is known that c-Fos induction from running persists after as many as 40 days of continuous access to exercise wheels (Clark et al., 2009), whether or not c-Fos expression attenuates for a given distance traveled over the course of access to wheels is unknown.

The objective of this study was to determine whether the proliferation and survival of new neurons formed in C57BL/6J mouse hippocampus is greater during the first days of voluntary running or after 21 days, when mice reach peak running distance. Further, the degree of granule cell activation (as measured by immunohistochemical detection of c-Fos) was measured at 4 independent time points during the course of voluntary running. The purpose was to determine the extent to which exercise-induced granule cell activation changes over the course of access to running wheels, as well as explore the relationship between granule cell activation and neurogenesis during prolonged wheel exposure.

The predicted outcome was not clear. On one hand, given that running distance is strongly correlated with survival of new hippocampal neurons and c-Fos expression (Oladehin and Waters, 2001, Rhodes et al., 2003a, Clark et al., 2009), it could be hypothesized that an increase in wheel running distance after several days may stimulate
neuron formation and activation to a greater extent. On the other hand, since cell proliferation during voluntary running and c-Fos activation following forced running have been reported to be reduced after several days (Lee et al., 2003b), the exercise-induced enhancement of neurogenesis and activation of the dentate gyrus may continue at a stable rate, or possibly decrease over time.

**Materials and methods**

*Animals and husbandry*

Two cohorts of male C57BL/6J mice arrived at the Beckman Institute Animal Facility from The Jackson Laboratory at 5 weeks of age (n=24) and 9 weeks of age (n=12). Upon arrival, mice were housed 4 per cage in standard polycarbonate shoebox cages with corncob bedding (Harlan Teklad 7097 ¼ inch, Madison, Wisconsin, USA) for 4 weeks (5 week old mice) or 3 weeks (9 week old mice). Subsequently, and for the remainder of the experiment, the mice were individually housed in either standard shoebox cages (without filter tops) or cages with running wheels attached.

Dimensions of running wheel cages were 36 x 20 x 14 cm (L W H) with a 23 cm diameter wheel mounted in the cage top (Respironics, Bend, OR). Cages without wheels were 29 x 19 x 13 cm (L W H). Wheel rotations were monitored continuously in 1 min increments throughout the experiments via magnetic switches interfaced to a computer running VitalView software (Respironics, Bend, OR). Rooms were controlled for temperature (21 ± 1°C) and photo-period (12:12 L:D; lights on at 7:00 AM and off at 7:00 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.

*Experimental Design*

Mice were randomly assigned to 6 groups (n=6 per group) as shown in Figure 1.1. All mice received 5 days of BrdU injections (50 mg/kg/day) starting when the mice were 12 weeks of age, to label dividing cells. Animals were either sampled 24 hrs after the last BrdU injection to measure cell proliferation and early neuronal differentiation (groups 1A, b and c), or after 25 days to measure survival of new neurons (groups 2a, b and c). In groups 1b and 2b, mice were individually housed in cages with access to wheels at the
time of BrdU injections, whereas in groups 1c, and 2c, mice were placed on wheels 21
days before the first BrdU injection. The inclusion of these groups (1c and 2c), where
animals had 21 days of wheel running before the BrdU injections, allowed us to
determine whether proliferation and survival of newly formed neurons is different at the
onset of wheel access, when levels of running are relatively low, as compared to after 21
days when running has reached peak levels. Group 1a and 2a represent the respective
sedentary controls.

All mice were euthanized 2.5 hours after the onset of the dark phase of the
light/dark cycle, which is approximately 1.5 hours after the peak of the daily wheel
running activity. Animals were sampled at this time to detect running-induced c-Fos
because peak expression of c-Fos protein is displayed approximately 90 minutes after cell
activation.

**Immunohistochemistry**

Animals were anesthetized with 150 mg/kg sodium pentobarbital (ip) and then
perfused transcardially with 4% paraformaldehyde in a phosphate buffer solution (PBS).
Brains were post-fixed overnight, and transferred to 30% sucrose in PBS. Brains were
sectioned using a cryostat into 40 micron coronal sections and stored in tissue
cryoprotectant at -20°C. Three separate 1-in-6 series of these sections (i.e., series of
sections throughout the rostro-caudal extent of the brain with 240 micron increments
separating each section) were stained in each of the following ways.

1) BrdU-DAB and Ki67-DAB. Purpose: Cell proliferation and mitotic markers. Free
floating sections were washed in tris-buffer solution (TBS) and then treated with 0.6%
hydrogen peroxide. To denature DNA for BrdU detection, sections were pre-treated with
50% de-ionized formamide, 10% 20XSCC buffer, 2N hydrochloric acid, 0.1 M Boric
acid. Sections were blocked with a solution of 0.3% Triton-X and 3% goat serum in TBS
(TBS-X plus), and then incubated in primary antibody against BrdU made in rat
(Accurate, Westbury, NY) at a dilution of 1:100 or Ki67 made in rabbit (Abcam,
Cambridge, MA) at 1:500 in TBS-X plus for 72 hrs at 4 ºC. 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) and stained using a diaminobenzidine kit (Sigma, St. Louis, MO).

2) Triple-fluorescent label. Purpose: To determine the proportion of BrdU-positive cells (BrdU+) in the dentate gyrus that differentiated into neurons. The same procedure as 1 was used except a cocktail was used for the primary antibody step, rat anti-BrdU (1:50; Accurate, Westbury, NY) and mouse anti-NeuN (1:50; Chemicon, Billerica, MA), together. Secondary antibodies made in goat were conjugated with fluorescent markers (Cy2-anti mouse, Cy3 anti-rat) at dilution 1:200 and also delivered as a cocktail.

3) c-Fos-DAB. Purpose: To detect acute neuronal activation. Free floating sections were washed in PBS and then treated with 0.6% hydrogen peroxide. Sections were then blocked with a solution of 0.2% Triton-X and 5% goat serum in TBS (TBS-X plus) for 1 hour, and then incubated in primary antibody against c-Fos made in rabbit (Calbiochem, San Diego, CA) at a dilution of 1:20,000 in PBS-X plus for 48 hrs at 4 ºC. Sections were then washed in PBS, treated with PBS-X plus for 60 min and then incubated in secondary antibody against rabbit made in goat at 1:500 in TBS-X plus for 90 min at room temperature. Sections were then treated using the ABC system (Vector, Burlingame, CA) and stained using a diaminobenzidine kit (Sigma, St. Louis, MO).

**Image analysis**

1) BrdU-DAB, Ki67-DAB and c-Fos-DAB. Following (Clark et al., 2008), the entire granule layer (bilateral), represented in the 1-in-6 series was photographed by systematically advancing the field of view of the Zeiss brightfield light microscope, and taking multiple photographs, via camera interfaced to computer, under 10X (total 100X) magnification. Positively labeled cells in these photographs were counted to generate unbiased estimates of total number of labeled cells (BrdU, Ki67, or c-Fos). Positively labeled cells located in the top layers of tissue were not included in these estimates. In addition, the total volume of the dentate gyrus represented in the series was measured so that the counts could be expressed per cubic micrometer dentate gyrus sampled.

2) Triple label. A confocal Leica SP2 laser scanning confocal microscope (using a 40X oil objective, pinhole size 81.35 μm) was used to determine the proportion of BrdU positive cells differentiated into neurons (NeuN+). Each BrdU positive cell in the granular layer (represented in the 1-in-6 series) was analyzed by focusing through the
tissue in the z-axis to establish co-labeling with NeuN. The number of new neurons per cubic micrometer per mouse was calculated as the number of BrdU cells per cubic micrometer multiplied by average proportion BrdU cell co-expressing NeuN for the designated group (groups as listed in Fig. 1.2)

**Statistical Analysis**

Data were analyzed using SAS, or R statistical software. In all analyses, P < 0.05 was considered statistically significant. The proportion of BrdU labeled cells in the granule cell layer that also expressed NeuN was analyzed by logistic regression. For these analyses, the deviance is reported in place of an F statistic.

Average daily running distance was compared between the early and late-running groups using two sample T-tests assuming equal variance. The number of proliferating cells and new neurons per mm$^3$ of dentate gyrus were compared between groups (shown in Fig. 1.2) using 1-way ANOVA. T-tests were used for posthoc analysis.

The number of c-Fos cells per mm$^3$ of dentate gyrus was compared between running and sedentary animals using a two sample T-test assuming equal variance. The number of c-Fos cells were compared at 6, 26, 30, and 50 days of running using ANCOVA with day as the classification factor, distance run as the continuous variable, and the interaction between distance and day. Pearson’s correlations between amount of running (km/hr) and number of cells expressing c-Fos were estimated using simple linear regression.

**Results**

**Wheel running**

Wheel running distance increased steadily for the first 20 days and thereafter maintained a plateau as has been reported previously by our laboratory in male C57BL/6J mice (Figure 2 in Clark et al., 2008 and Figure 1 in Clark et al., 2009). During the 5 BrdU injection days, mice in groups 1b and 2b ran approximately half as far as mice in groups 1c and 2c (Fig. 1.2A) [$t(22) = 3.21, P = 0.003$]. Average distance traveled over the entire period following the initiation of BrdU injections was similar in group 2b as compared to 2c (Fig. 1.2B). The average level of wheel running over the entire experiment for all mice was 4.56 Km/day ($\pm 0.10$ S.E.).
 Neurogenesis

Cell proliferation.

The number of proliferating BrdU-labeled cells measured 24 hrs after the last injection did not differ across groups (Fig 1.3A). However, running increased the number of cells labeled with Ki67 in the subgranular zone of the dentate gyrus (Fig. 1.3B) \( F(2,15) = 10.00, P = 0.001 \). Post hoc analysis revealed that animals running for 6 days (group 1b) had greater numbers of Ki67 cells as compared to sedentary animals (group 1a) \( t(15) = 4.44, P = 0.001 \) or animals sampled on day 26 of running (group 1c) \( t(15) = 2.65, P = 0.01 \). Mice running for 26 days displayed slightly greater numbers of Ki67 cells than sedentary mice, however this difference was not statistically significant \( t(15) = 2.65, P = 0.09 \).

Early determination.

Running increased the proportion of BrdU cells double labeled with the neuronal marker NeuN, 24 hr after the 5th day of BrdU injections \( \text{deviance}(1,16) = 5.37, P = 0.02 \). The percentage of BrdU cells co-labeled with NeuN, 24 hrs after the last injection was 17.8% (±2.1) during the first 5 days of running, 18.6% (±1.4) during days 20-25 of running, and 12.6% (±2.9) in sedentary mice. This difference in proportion of differentiated cells led to an overall net increase in the total number of BrdU positive cells co-expressing NeuN in runners as compared to sedentary mice (Fig. 1.3C) \( F(2,15) = 9.80, P = 0.002 \). Post hoc analysis revealed that both runner groups 1b and 1c were different from sedentary but did not differ from each other.

Cell survival.

Running nearly doubled the number of surviving neurons 25 days after the last injection of BrdU independent of whether those neurons were formed during the first 5 days of running, or 20 days later when mice reached peak running performance (Fig. 1.3D) \( F(2,15) = 29.59, P < 0.0001 \). Survival of new neurons was marginally greater during the first 5 days of running (group 2b) as compared to during days 21-25 (group 2c), however this difference was not statistically significant \( t(15) = 1.98, P = 0.07 \). Runners displayed a significantly greater proportion of BrdU cells co-labeled with NeuN \( \text{deviance}(1,16) = 20.6, P < 0.0001 \). The percentage of BrdU cells double labeled with
NeuN 25 days after the last injection was 92.8% (±0.67) in group 2b, 92.3% (±0.72) in peak runners, group 2c, and 81.7% (±1.2) in sedentary mice, group 2a. 

**c-Fos induction from wheel running**

Runners displayed significantly greater numbers of c-Fos positive cells in the granular layer of the dentate gyrus as compared to sedentary animals (Fig. 1.4A, B) \[t(32) = 3.96, P = 0.0003\]. Distance run 90 min before euthanasia was significantly correlated with numbers of c-Fos positive cells among all individuals sampled on day 6, 26, 30, and 50 (Fig. 1.4C) \[F(1,16) = 89.15, P < 0.0001\]. Separated by group, the Pearson’s correlation (r) was 0.96, 0.88, 0.92, and 0.87 for day 6, 26, 30, and 50 respectively (all P≤ 0.02). The number of c-Fos positive cells for an equivalent distance significantly differed according to day, as reflected by a significant interaction between day and distance run (Fig. 1.4C) \[F(3,16) = 3.20, P = 0.05\]. Post-hoc analysis revealed that mice sampled on day 6 displayed a significantly steeper slope than all other days (Fig. 1.4C) \[t(16), all P < 0.02\]. The slopes for animals sampled on days 26, 30, and 50 were less steep and not significantly different from each other.

**Discussion**

The main finding of this study is that proliferation (Fig. 1.3B) of progenitor cells in the dentate gyrus is significantly reduced after 21 days of running compared to after 5 days even though running is approximately twice as high after 21 days. Despite reduced proliferation during escalation of running, net neurogenesis was maintained at a relatively stable level via increased differentiation (Fig. 1.3C) and survival (Fig. 1.3D). The explanation for why proliferation is higher at the onset is unclear but may be related to the novelty of the experience, initial learning components, or stress regulation. Results also extend and confirm recent studies showing that increased survival and neuronal differentiation, not proliferation, maintain high rates of neurogenesis above sedentary during sustained wheel running (Kronenberg et al., 2006, Fuss et al., 2009, Snyder et al., 2009b).

In a previous study, we found that the number of surviving neurons born during the onset of running (first 10 days) is strongly correlated with both average distance run during BrdU injections and total distance run over the course of the study in the C57BL/6J model (Clark et al., 2009). If distance run during BrdU injections was the
primary factor in determining the amount of new neurons, then we would expect that cells labeled later, when levels of running were higher, would yield a greater number of new neurons. However, our data do not support that prediction. Although running distance nearly doubled after 20 days of wheel access as compared to the first 5 days (Fig. 1.2A), the survival of new neurons labeled later, during higher levels of running, displayed a slight, statistically non-significant, reduction as compared to earlier when levels of running were lower (Fig 1.3C & D). Cell division displayed an even stronger decrease over the same period, as the number of Ki67 positive cells returned to the level of sedentary animals after 25 days of running (Fig. 1.3B). These results are consistent with recent studies suggesting that sustained running maintains high rates of neurogenesis above sedentary levels primarily by increasing survival as opposed to proliferation of dividing cells (Kronenberg et al., 2006, Fuss et al., 2009, Snyder et al., 2009b). It is notable that this is contrary to initial reports suggesting that increased proliferation was the dominant factor contributing to increased neurogenesis from voluntary running (van Praag et al., 1999b).

The current study is the first to show that not even a doubling in wheel running distance during prolonged access to running wheels can restore proliferation to levels similar to that during the initial days of running. Taken together with recent reports, these data suggest that the contribution of increased mitotic activity to net increases in numbers of new neurons induced from running is probably small, and that the major factor is probably changes in differentiation and survival (Kronenberg et al., 2006, Fuss et al., 2009, Snyder et al., 2009b). Notably, we found no increase in the number of BrdU cells when assessed 24 hrs after the last injection at the onset of access to running wheels or during peak running (Fig 1.3A), even though we observed a significant increase in differentiation toward the neuronal lineage (Fig. 1.3C), and an approximate doubling in survival of new neurons after 25 days (Fig. 1.3D).

One plausible explanation for the discrepancy between BrdU (Fig. 1.3A) and Ki67 (Fig. 1.3B) is that many of the cells dividing during the first few days of running could be quickly dying off, thereby reducing the number of recently divided BrdU cells to similar levels as sedentary mice. A recent study reported an increase in cells undergoing apoptosis in the dentate gyrus at day 7 of wheel running in the C57BL/6
mouse (Kitamura and Sugiyama, 2006). The majority of the cells undergoing apoptosis were located in the sub-granular zone, where neuronal progenitor cells proliferate. Studies have also demonstrated that wheel running increases cell proliferation in the sub-granular zone in a step-wise manner daily, peaking between 10-17 days in C57BL/6 mice (Kitamura and Sugiyama, 2006, Kronenberg et al., 2006, Van der Borght et al., 2009). Moreover, several reports have either shown a subtle (not statistically significant, P>0.05) increase in cell proliferation, or no increase when examining various points between 2 and 7 days of running (van Praag et al., 2002, Kitamura and Sugiyama, 2006, Van der Borght et al., 2009). Thus, by day 5 of running, it is possible that the number of proliferating cells had not yet reached peak amounts. Given that increased cell division has not reached a maximum and the possibility of increased apoptosis at the onset of running, it is probable that during the first few days of running many of the Ki67 cells undergoing mitosis quickly die off following division within the first 24 hours. This could return the total number of early surviving proliferating cells (as measured by BrdU 24 hours after the last injection) in running mice to levels similar to that of sedentary animals (Fig 1.3a).

Results of this study (Fig. 1.3C) confirm previous reports showing that neuronal differentiation begins within the first 5 days of birth of new neurons (Kempermann et al., 2003). In this study, 12-18% of BrdU cells 24 hours after the last BrdU injection displayed NeuN. Wheel running is well known to increase the proportion of BrdU labeled cells that co-express NeuN at approximately day 40 of the cell’s life (van Praag et al., 1999b, Clark et al., 2008). The present study extends these results for 6 day old BrdU cells.

In this study we used c-Fos to measure neuronal activation induced from acute wheel running. Typically, c-Fos habituates after repeated exposure to a stimulus (e.g., restraint stress, novel environment, immune stimulation, or drug exposure, Umemoto et al., 1994, Curran et al., 1996, Struthers et al., 2005, Kohman et al., 2009). Running is an unusual c-Fos-evoking stimulus, as the up regulation of c-Fos in the dentate gyrus is known to persist after as many as 40 days of continuous access to running wheels (Clark et al., 2009), and in the present study 50 days (Fig. 1.4C). In response to mild forced exercise on a treadmill consisting of walking at a speed of 0.48 km/hr in rats, c-Fos
protein expression in the dentate gyrus increased during the initial days, then started to decrease by day 7 before reaching a plateau at a middle level after 14 days of this training (Lee et al., 2003b). This is generally consistent with our results for voluntary wheel running in mice where the c-Fos response was slightly greater on day 6 of running as compared to the other later days. Taken together this suggests that the initial increase in c-Fos in our study may not be related to the escalation in running because in the treadmill study the level of exercise was maintained at a constant rate during training (Lee et al., 2003b). The explanation for why c-Fos is slightly higher on day 6 for a given level of running is therefore unclear but may be related to the novelty of the experience, initial learning components, or stress regulation.

The degree of c-Fos induction from acute bouts of running occurred in parallel with rates of mitosis and survival of new cells over the course of exercise training. Running-induced c-Fos expression was slightly elevated during the initial days of training, a time that corresponded with peak levels of cell mitosis (Fig. 1.3C) and a slight increase in cell survival (Fig. 1.3C & D). With continued running, decreased proliferation decreased survival of new neurons paralleled the slightly attenuated c-Fos response to wheel running (Fig. 1.4C).

The parallels between acute c-Fos induction from running and adult hippocampal neurogenesis in this study may be related to “activity-sensing” properties of neuronal progenitor cells (Deisseroth et al., 2004). Excitation of progenitor cells, in vitro, increases differentiation into the neuronal lineage (Deisseroth et al., 2004). The location of neural progenitor cells in the subgranular zone, situated between the granule cell bodies and the axon-rich polymorphic layer, would place progenitor cells in a niche ideal for sensing increased granular activity from wheel running. The relationship between granule cell activation and neurogenesis suggests that neuronal excitation created by running may contribute to the induction of new neuron development in the hippocampus (Clark et al., 2009).

Recent work has implicated the neurotransmitter gamma-Aminobutyric acid (GABA) in the activity-dependent differentiation of progenitor cells into new neurons (Markwardt and Overstreet-Wadiche, 2008). GABA interneurons are the first cell type to establish connections with new born granule cells (Overstreet Wadiche et al., 2005, Ge et
al., 2006). Due to intracellular differences in ion concentrations between mature neurons and progenitor cells, GABA has an excitatory effect on progenitor cells (Owens and Kriegstein, 2002). The unusual excitatory role of GABA on progenitor cells has been suggested to be important for promoting factors necessary for neuronal differentiation (e.g. NeuroD, Tozuka et al., 2005). Many hippocampal glutamatergic mossy fibers and theta rhythm regulating medial septum projections also innervate GABAergic interneurons in the dentate gyrus (Bilkey and Goddard, 1985, Szabadics and Soltesz, 2009). These connections may provide the necessary stimulation of dentate GABAergic interneurons to promote neurogenesis during events that increase hippocampal activity, such as wheel running.

In conclusion, we report that long term wheel running maintains a relatively constant rate of adult hippocampal neurogenesis above sedentary levels that decreases slightly (but not significantly), after 20 days relative to during escalation in the C57BL/6J mouse model. These results, in conjunction with other recent reports (Kronenberg et al., 2006, Fuss et al., 2009, Snyder et al., 2009b), suggest that net increases in adult hippocampal neurogenesis from sustained wheel running are likely a result of increased survival and neuronal differentiation, not proliferation. The functional significance of increased neurogenesis and activity dependent regulation, whether to replenish a population of dying cells, or build and maintain a larger dentate gyrus and significance, if any, in behavior, remain important topics for future investigations.
Figure 1.1. Experimental design. Group 1 was used to measure proliferation and group 2 survival of new neurons born in non-running controls (sub-group a), at the onset of access to running wheels (sub-groups b), and 20 days after the onset of access to wheels during peak running distance (sub-groups c). All mice were 12 weeks old at the onset of BrdU injections and the same age at euthanasia. Open boxes represent periods in which the animals were individually housed in standard laboratory cages without access to running wheels. Boxes with horizontal stripes represent periods during which the animals were individually housed with free access to running wheels.
Figure 1.2. Average wheel running distance between groups. A) Average daily running distance in km/day (±SE) over 5 days of BrdU injections shown separately for the group given BrdU injections at the onset of access to running wheels versus after 20 days of running. B) Average daily running distance in km/day (±SE) over 30 days starting at the onset of BrdU injections until euthanasia. The x-axis represents groups as shown in Figure 1.1. $$ indicates p<0.01.
Figure 1.3. Proliferation, mitosis, early differentiation and survival of new neurons. A) 
*Top:* Representative coronal section of the dentate gyrus stained for BrdU-DAB, 24 hrs 
after the last BrdU injection. *Bottom:* Average number of BrdU labeled cells (±SE) per 
cubic mm in the granular layer of the dentate gyrus, measured 24 hours after the 5th
BrdU injection. B) Top: Representative coronal section of the dentate gyrus stained for Ki67-DAB on day 6 of running. Bottom: Average number of Ki67 labeled cells (±SE) per cubic mm in the sub-granular zone. C) Top: Representative coronal section of the dentate gyrus stained for BrdU (red) and NeuN (green), 24 hrs after the last BrdU injection. The arrows indicate double labeled cells. Bottom: Average number of BrdU labeled cells co-expressing NeuN (±SE) per cubic mm in the granular layer of the dentate gyrus, 24 hours after the 5th BrdU injection. D) Top: Representative coronal section of the dentate gyrus stained for BrdU (red) and NeuN (green), 25 days after the last BrdU injection. Bottom: Average number of BrdU labeled cells co-expressing NeuN (±SE) per cubic mm in the granular layer of the dentate gyrus 25 days after the 5th BrdU injection. Sedentary animals are shown as white bars, animals that received BrdU during the first 5 days of running are shown as grey bars, and animals that received BrdU during days 21-25 of running are shown as black bars. The x-axis of each graph represents groups as shown in Figure 1.1. *** indicates p<0.001 different from sedentary, **p<0.01 different from sedentary, $$p<0.01$$ different from days 1-5 of BrdU injections, not statistically different from sedentary.
Figure 1.4. c-Fos induction from wheel running.  A) Representative coronal section stained for c-Fos-DAB (combined with a light Nissl stain to highlight the dentate gyrus) of an animal euthanized in the sedentary condition (50X total magnification).  B) Same as A for an animal running before euthanasia.  C) Number of c-Fos positive cells per cubic mm shown for individual mice sampled on day 6 (closed black diamonds), day 26 (closed dark grey squares), day 30 (closed light grey triangles), and day 50 (open circles) plotted against distance run in km within 90 minutes before euthanasia. Day 6 had a significantly steeper slope than all other days sampled.
References


CHAPTER 2

Functional analysis of neurovascular adaptations to exercise in the dentate gyrus of young adult mice associated with cognitive gain.

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Abstract
The discovery that aerobic exercise increases adult hippocampal neurogenesis and can enhance cognitive performance holds promise as a model for regenerative medicine. This study adds two new pieces of information to the rapidly growing field. First, we tested whether exercise increases vascular density in the granular layer of the dentate gyrus, whole hippocampus, and striatum in C57BL/6J mice known to display pro-cognitive effects of exercise. Second, we determined the extent to which new neurons from exercise participate in the acute neuronal response to high levels of running in B6D2F1/J (F1 hybrid of C57BL/6J female by DBA/2J male). Mice were housed with or without a running wheel for 50 days (runner versus sedentary). The first 10 days, they received daily injections of BrdU to label dividing cells. The last 10 days mice were tested for performance on the Morris water maze and rotarod and then euthanized to measure neurogenesis, c-Fos induction from running and vascular density. In C57BL/6J, exercise increased neurogenesis, density of blood vessels in the dentate gyrus and striatum (but not whole hippocampus) and enhanced performance on the water maze and rotarod. In B6D2F1/J, exercise also increased hippocampal neurogenesis but not vascular density in the granular layer. Improvement on the water maze from exercise was marginal, and no gain was seen for rotarod, possibly due to a ceiling effect. Running increased the number of c-Fos positive neurons in the granular layer by 5-fold, and level of running was strongly correlated with c-Fos within 90 minutes before euthanasia. In runners, approximately 3.3% (± 0.008 S.E.) of BrdU positive neurons in the middle of the granule layer displayed c-Fos as compared to 0.8% (± 0.001) of BrdU negative neurons. Results suggest pro-cognitive effects of exercise are associated with increased vascular density in the dentate gyrus and striatum in C57BL/6J mice and that new neurons from exercise preferentially function in the neuronal response to running in B6D2F1/J.
Introduction

An acute bout of aerobic physical activity, whether voluntary or forced, strongly and selectively increases neuronal activity in the hippocampus (Vanderwolf, 1969, Oddie and Bland, 1998, Bland et al., 2006). This is true even when animals are running in place, such as on a running wheel or on a treadmill (Hirase et al., 1999, Oladehin and Waters, 2001). It thus appears to occur independently from changes in place or spatial information (Bose and Recce, 2001, Bland et al., 2006). For example, the speed of voluntary wheel running is strongly correlated with the discharge frequency of pyramidal cells and interneurons in the hippocampus of rats (Czurko et al., 1999). In outbred Hsd:ICR mice, running increases c-Fos in the dentate gyrus by 3-fold. Moreover, the level of c-Fos induction is strongly correlated with the average speed of wheel running within the 90 minutes preceding euthanasia (Rhodes et al., 2003a).

Aerobic physical activity (either voluntary or forced) also massively increases neurogenesis in the dentate gyrus of mice and rats (van Praag et al., 1999b, Rhodes et al., 2003b, Uda et al., 2006, Leasure and Jones, 2008, Trejo et al., 2008). This results in increased total numbers of granular neurons (Rhodes et al., 2003b) and increased volume of the entire granule layer (Rhodes et al., 2003b, Clark et al., 2008). This discovery has generated great excitement because it represents a model for understanding how an effective regenerative system works in the adult mammalian brain (Cotman et al., 2007). The present study aims to fill 2 gaps in the rapidly growing field.

It is known that adult hippocampal neurogenesis occurs in the inside layer of the dentate gyrus adjacent to the hilus, near blood vessels and it has been hypothesized that exercise increases angiogenesis in this area to support neurogenesis (Palmer et al., 2000, Pereira et al., 2007). However, studies reporting direct measurements of angiogenesis or vascular density in the granular layer of the dentate gyrus in response to exercise are scarce. Although it is established that exercise increases vascular density in the motor cortex (e.g., Swain et al., 2003), cerebellum (e.g., Black et al., 1990) and striatum (e.g. Ding et al., 2006b), to the best of our knowledge, only three studies have reported changes in properties of blood vessels in the dentate gyrus in response to exercise. In the first study, van Praag et al. (2005) counted blood vessel fragments within sections of the whole dentate gyrus, and collected measurements on 2 individual vessel fragments per
animal using Lectin stain. They found that voluntary wheel running did not change the number of fragments but increased surface area and perimeter of the individual vessels sampled in young but not old male C57BL/6J mice. In a later study by the same group, they measured the percentage of the whole dentate gyrus covered by blood vessels in young animals housed with wheels for only 2 hrs per day (versus animals never housed with wheels) and they found no difference between groups (van Praag et al., 2007). In the third study, Ekstrand et al. (2008) counted number of cells in the molecular layer of the dentate gyrus co-labeled with both BrdU and rat-endothelial cell antigen-1 (indicating new vascular tissue) in adult, male, Wistar rats. They found approximately a threefold increase in the numbers of such cells (i.e., angiogenesis) in animals housed individually with running wheels as compared to single housed sedentary controls. Taken together these data suggest that exercise likely increases the area covered by vessels in the dentate gyrus, but a direct measurement of area fraction, specifically in the granular layer of the dentate gyrus (where neurogenesis occurs), to our knowledge, has not been reported.

Another gap in the literature relates to the functional significance of adult hippocampal neurogenesis (Kempermann, 2008). Recent studies have established that new neurons generated in adulthood display morphological and electrophysiological properties consistent with older granular neurons generated during early postnatal development (van Praag et al., 1999a, van Praag et al., 2002, Laplagne et al., 2006, Zhao et al., 2006, Toni et al., 2008). Moreover, several studies have reduced neurogenesis using a chemical toxin (e.g., Shors et al., 2002), irradiation (e.g., Meshi et al., 2006, Saxe et al., 2006, Clark et al., 2008) or genetic engineering approaches (e.g., Dupret et al., 2008) to see whether blockade of neurogenesis impairs learning and memory on behavioral tasks hypothesized to require hippocampus. However, results are mixed, and depend on the technique used to reduce neurogenesis, the behavioral task examined, genotype or species, as well as the timeframe between when neurogenesis is interrupted and behavior measured.

To circumvent some of these issues, a few recent studies have examined whether the new neurons are preferentially recruited into neuronal responses to a water maze experience (Jessberger and Kempermann, 2003, Kee et al., 2007b) or repeated exposure to an enriched environment (Tashiro et al., 2007) as compared to older neurons using
immunohistochemical detection of immediate early gene expression (e.g., c-Fos). The data suggest that there is a critical period when new neurons are more likely to show c-Fos induction than older neurons. However, relatively few scattered granule neurons display c-Fos immediate early gene activity in response to the water maze (Jessberger and Kempermann, 2003) or re-exposure to an enriched environment (Tashiro et al., 2007). In comparison, a large number of neurons display c-Fos in response to wheel running (Rhodes et al., 2003a). It is not clear why such a large neuronal activation of the dentate gyrus takes place in response to acute bouts of wheel running. Nonetheless, an alternative hypothesis is that the new neurons are recruited into the function of the hippocampus in the neuronal response to wheel running itself (Rhodes et al., 2003b).

This study had two main objectives. The first was to determine the extent to which the volume fraction of blood vessels in the granular layer of the dentate gyrus, whole hippocampus, and whole striatum changes after chronic voluntary wheel running exercise in C57BL/6J mice. C57BL/6J mice were chosen for this analysis because they are well known to display enhanced behavioral performance from wheel running exercise and hence serve as a useful model to explore neurobiological correlates of cognitive enhancement (van Praag et al., 1999a, Clark, 2008, van Praag et al., 2005). We predicted volume fraction of blood vessels would increase in all three areas.

The second objective was to determine the extent to which new neurons from exercise are recruited into the acute neuronal c-Fos induction from wheel running in the granular layer of the dentate gyrus. For this objective, we chose to study B6D2F1/J mice as opposed to C57BL/6J because the F1 hybrid mice run at higher levels and previous studies have established that level of running within 90 minutes prior to euthanasia is positively correlated with amount of c-Fos induction (Rhodes et al., 2003a). Moreover, differences in chronic levels of running are positively correlated with number of new neurons (Rhodes et al., 2003b). Therefore, we reasoned that by virtue of their higher levels of running, F1 hybrid mice would show larger numbers of new neurons and larger numbers of c-Fos positive cells than C57BL/6J, which would increase the probability of finding overlap between the two. We predicted that new neurons would be preferentially recruited into the neuronal response to wheel running in this strain (i.e., that BrdU positive neurons would display greater probability for c-Fos induction from running than
BrdU negative neurons). To the best of our knowledge, B6D2F1/J mice have not been measured for effects of exercise on the water maze or rotarod. Therefore another objective of this experiment was to test the extent to which the F1 strain displays benefits of exercise on behavioral tasks. Here the prediction was not as clear. On the one hand, given that they run more than C57BL/6J, they would be expected to show higher levels of neurogenesis, and perhaps stronger pro-cognitive effects. On the other hand, previous studies using mice selectively bred for high levels of wheel running found that the high runners did not display gains in performance from running even though they ran approximately 12 km/day and generated more neurogenesis than the unselected control lines that ran 4 km/day (Rhodes et al., 2003b).

Materials and Methods

Animals

Mice from the C57BL/6J strain (n=24 males) and B6D2F1/J strain (n=6 females and 6 males) were used. B6D2F1/J mice are the hybrid offspring of a cross between a C57BL/6J female and a DBA/2J male. Individual animals within this strain share the feature with C57BL/6J that they are isogenic (meaning that all same sex members are genetically identical). However, they are heterozygous at all loci that differ between DBA/2J and C57BL/6J, and hence they have the potential to display what has been referred to as “hybrid vigor”, or enhanced performance (Livesay, 1930, Han et al., 2008).

Husbandry

Animals arrived at the Beckman Institute Animal facility from The Jackson Laboratory at 5 wks of age. Upon arrival they were housed 4 per cage by sex in standard polycarbonate shoebox cages with corncob bedding (Harlan Teklad 7097 ¼ inch, Madison, Wisconsin, USA). They were housed this way for 11 (Experiment 1) or 14 days (Experiment 2) 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 (12:12 L:D; lights on at 7am and off at 7pm). 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.
Experiment 1: C57BL/6J males (n=24)

The primary objective of this experiment was to determine the extent to which the volume fraction of blood vessels in the granular layer of the dentate gyrus, whole hippocampus, and striatum changes after chronic voluntary wheel running exercise in young male C57BL/6J mice. A secondary objective was to confirm that the vascular changes were associated with increased hippocampal neurogenesis and enhanced behavioral performance on the water maze, as previously reported for this strain (van Praag et al., 1999a, van Praag et al., 2005).

Mice (46 days old) were placed individually in cages either without (Sedentary) or with running wheels (Runners) for 50 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 and we wanted to keep physical activity to a minimum in the sedentary group (Koteja et al., 1999, Rhodes et al., 2000). Dimensions of running wheel cages were 36 x 20 x 14 cm (L W H) with a 23 cm diameter wheel mounted in the cage top (Respironics, Bend, OR). Dimensions of cages without wheels were 29 x 19 x 13 cm (L W H). Wheel rotations were monitored continuously in 1 min increments throughout the experiments via magnetic switches interfaced to a computer running VitalView software (Respironics, Bend, OR).

After 36 days of being housed with or without wheels, mice (age 82 days) were tested on 2 behavioral tasks, Morris water maze, then rotarod. Testing took place during the light phase of the light/dark cycle. 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.

Water maze

Mice were trained on Morris water maze with 2 trials per day for 5 days. A trial lasted either 60 sec or after the mouse reached the platform and remained on the platform for 10 sec. If a mouse did not reach the platform in 60 sec it was gently guided there by hand. Mice were placed back in their cage and allowed to rest for 30 sec between trials. One hour after training on day 5, the platform was removed and mice were tested with a probe trial (60 sec).
The maze consisted of a circular tub, 150 cm diameter and 30 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. 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 vide tracking from above (black mouse on white background). Water temperature was maintained at 25-26 °C. Topscan (CleverSystems, Reston, VA) 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). 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 4 consecutive trials per day for 3 days.

Experiment 2: B6D2F1/J (n=12; 6 males and 6 females)

The primary objective of this experiment was to determine the extent to which new neurons from exercise are recruited into the neuronal c-Fos response to running in the dentate gyrus. A secondary objective was to determine the extent to which exercise changes morphology of the dentate gyrus (vascular density and neurogenesis) and behavioral performance in F1 mice.

The experiment proceeded in exactly the same way as experiment 1 except for the following. Mice were 49 days old instead of 46 days old when they were placed individually with or without running wheels. Mice were tested for behavioral performance after 42 (rather than 36) days of running. A smaller water maze was used (100 cm rather than 150 cm), with the same size platform (8.5 x 8.5 cm). The probe trial was given 24 hours (rather than 1 hour) after the final acquisition trial. The following day after the probe trial, the mice were trained on a visible platform version of the water maze (2 trials per day for 3 days). The trials proceeded similar to the hidden version except a flag pole (19.5 cm) rose from the middle of the platform bearing a flag (black plastic) and the platform was moved to the middle of a different, randomly selected
quadrant each trial (excluding the target quadrant from the hidden version). After behavioral testing, the mice were given 2 days to recover levels of wheel running. Then, they were euthanized at the height of their active period, between 2 and 3 hours after the lights shut off in the animal rooms.

**Immunohistochemistry**

Following Clark et al. (2008), animals were anesthetized with 100 mg/kg sodium pentobarbital (ip) and then perfused transcardially with 4% paraformaldehyde in phosphate buffer solution (PBS). Brains were postfixed overnight, and transferred to 30% sucrose in PBS. Brains were sectioned using a cryostat into 40 micron coronal sections and stored in tissue cryoprotectant at -20°C. Four separate 1-in-6 series of these sections (i.e., series of sections throughout the rostro-caudal extent of the brain with 240 micron increments separating each section) were stained in each of the following ways.

1) BrdU-DAB. Purpose: To detect BrdU-positive (newly divided) cells in the dentate gyrus. Free floating sections were washed in tissue buffering solution (TBS) and then treated with 0.6% hydrogen peroxide. To denature DNA, sections were treated with 50% de-ionized formamide, 10% 20XSCC buffer, 2N hydrochloric acid, 0.1 M Boric acid. Sections were then treated with a solution of 0.3% Triton-X and 3% goat serum in TBS (TBS-X plus), and then incubated in primary antibody against BrdU made in rat (Accurate, Westbury, NY) at a dilution of 1:100 in TBS-X plus for 72 hrs at 4 ºC. 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) and stained using a diaminobenzidine kit (Sigma, St. Louis, MO).

2) c-Fos-DAB. Purpose: To detect c-Fos positive (transcriptionally activated) cells in the dentate gyrus in response to wheel running. Free floating sections were washed in PBS and then treated with 0.5% hydrogen peroxide. Sections were then treated with a solution of 0.2% Triton-X and 5% goat serum in TBS (TBS-X plus) for 1 hour, and then incubated in primary antibody against c-Fos made in rabbit (Calbiochem, San Diego, CA) at a dilution of 1:20,000 in PBS-X plus for 48 hrs at 4 ºC. Sections were then washed in PBS, treated with PBS-X plus for 60 min and then incubated in secondary antibody against rabbit made in goat at 1:500 in TBS-X plus for 90 min at room temperature.
Sections were then treated using the ABC system (Vector, Burlingame, CA) and stained using a diaminobenzidine kit (Sigma, St. Louis, MO).

3) Collagen IV-DAB. Purpose: To visualize blood vessels in brain sections. Following Franciosi et al. (2007), free floating sections were washed in PBS, followed by distilled water at 37°C. Sections were then treated with pepsin (1 mg/ml) in 0.2N HCL at 37 ºC for 12 min for antigen retrieval, then washed in PBS for 15 min at 27 ºC. Sections were then transferred to TBS, treated with 0.5% hydrogen peroxide in TBS for 30 min, washed again in TBS and then treated with 0.1% Triton-X and 3% goat serum in TBS. Sections were then incubated in primary antibody against collagen IV at a dilution 1:300 in TBS-X plus for 72 hours. Following incubation, sections were washed in TBS, treated with TBS-X plus for 1 hour, and then incubated in secondary antibody against rabbit made in goat at 1:500 in TBS-X plus for 90 minutes. Finally, sections were treated using the ABC system (Vector, Burlingame, CA) and stained using a diaminobenzidine kit (Sigma, St. Louis, MO). Once mounted on slides, sections were lightly stained with methylene blue (1000 ml water, 0.16g Methylene Blue, 0.1g Azure II, 1.7g Na2HPO4, 0.56g KH2PO4).

4) Triple-fluorescent label. Purpose: Experiment 1. To determine the proportion of BrdU-positive cells (BrdU+) in the dentate gyrus that differentiated into neurons. Experiment 2. To determine the proportion of BrdU+ and BrdU-negative (BrdU-) neurons in the dentate gyrus that expressed c-Fos. The procedure for BrdU-DAB was repeated except for the following. A cocktail was used for the primary antibody step, rat anti-BrdU (1:50; Accurate, Westbury, NY), mouse anti-NeuN (1:50; Chemicon, Billerica, MA), and rabbit anti-c-Fos (1:7000; Calbiochem, San Diego, CA). Secondary antibodies made in goat were conjugated with fluorescent markers (Cy2 anti-rabbit, Cy3 anti-rat, Cy5-anti mouse) at dilution 1:200 and also delivered as a cocktail. ABC and diaminobenzidine steps were omitted.

Image analysis
1) BrdU-DAB and c-Fos-DAB. Following Clark et al. (2008), the entire granule layer (bilateral), represented in the 1-in-6 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 10X (total 100X) magnification.
These photographs were then analyzed using ImageJ software to generate unbiased estimates of total number of labeled cells (BrdU or c-Fos) per cubic micrometer dentate gyrus sampled and area of the granular layer within the sections.

2) Blood vessels. The entire striatum, hippocampus, and dentate gyrus represented in a 1 in 6 series, unilaterally, was photographed by systematically advancing the field of view of the Zeiss brightfield light microscope under 20X (total 200X) magnification. A random sample of these photographs (12 per individual for dentate gyrus and 20 for striatum and whole hippocampus) was then microanalyzed to estimate vascular density. A 494 point grid was placed over each of these photographs using ImageJ software. The area of interest was outlined. Then, the total number of grid intersections that crossed over a blood vessel and the total number of grid intersections within the outlined region were counted manually and expressed as a ratio, equivalent to the area fraction (Sikorski et al., 2008). The measurements were repeated for 8 individuals (using a different sample of photographs) to establish repeatability of individual measurements and measurement error. In addition, the area of the structures within sections was estimated by outlining the structures using Stereo Investigator software (version 7.5, MBF Bioscience, Williston VT).

3) Triple label. A confocal Leica SP2 laser scanning confocal microscope (using a 40X oil HCX PL APO C5 objective with 1.25 numerical aperture, pinhole size 81.35 μm, 1-Air Unit) was used to determine the proportion of BrdU positive cells differentiated into neurons (NeuN+) (in both experiments) and to determine the proportion of neurons (BrdU+ or BrdU-) displaying c-Fos (in Experiment 2). Each BrdU positive cell in the granular layer (represented in the 1-in-6 series) was microanalyzed by performing sequential line scans (line average 8) to establish co-labeling with NeuN or c-Fos. In addition, the granular layer represented in the 1-in-6 series was focused on a single plane (randomly selected on the z-axis), and all the granular neurons (not labeled with BrdU, i.e., BrdU-) were counted as well as the number co-labeled with c-Fos in that plane (BrdU-, NeuN+, c-Fos +).

Statistical analysis

In Experiment 1, we estimated the proportion of BrdU positive cells in the granular layer that also expressed NeuN mature neuronal marker. In experiment 2, we
estimated the proportion of BrdU positive or negative granular neurons co-labeled with c-Fos. These proportion data were analyzed among the groups by logistic regression. For these analyses the deviance is reported in place of the F statistics.

The sedentary group was compared to the runner group within strains using unpaired t-tests. In experiment 2, data were also analyzed using ANOVAs with sex, exercise treatment, and the interaction between sex and treatment as factors.

The water maze acquisition data and the rotarod data were analyzed within strains, using repeated measures ANOVA with day or trial as the within-subjects factor and exercise treatment (runner vs. sedentary) as the between-subjects factor. In experiment 2, sex and interactions between sex and treatment were also included in the analysis.

Pearson’s correlations between amount of running (e.g., km/hr or km/day) and number of c-Fos positive cells or number of new neurons were analyzed using simple linear regression.

Results

Wheel Running

C57BL/6J males ran approximately half as far as B6D2F1/J males or females [Fig. 2.1; \(F(1,15)=19.3, P<0.001\)]. Running was similar between the sexes in B6D2F1/J. Average level of wheel running over the entire experiment was 3.8 km/day (± 0.32 S.E.) in C57BL/6J males, whereas it was 8.1 (± 0.54) and 8.4 (± 0.71) km/day for B6D2F1/J males and females, respectively. Inspection of Figure 2.1 shows that in both genotypes, wheel running distance increased steadily for the first 20 days and thereafter maintained a plateau until behavioral testing when levels dropped by approximately 50%.

Experiment 1: Neurovascular adaptations to exercise in C57BL/6J mice.

The collagen IV-DAB immunohistochemical procedure consistently stained vasculature in the brain across all regions and all individuals (Fig. 2.1). The sampling method (Fig. 2.1B; 12 photographs per animal for the dentate gyrus and 20 for the striatum and whole hippocampus) produced negligible measurement error. This was established by repeating the measurements for 8 individuals (using a different sample of photographs). The Pearson’s correlation between duplicate measurements was greater than 0.98 for all three regions (all \(P<0.0001\)).
Dentate gyrus

Wheel running increased the number of new neurons twofold (Fig 2.3A) \([t(22)=4.3, P=0.0003]\). Level of running was significantly correlated with number of new neurons among individuals whether running was expressed as the total distance during the first 10 days \(R^2=0.36, n=12, P=0.04\), or total distance over the entire study (Fig. 2.3B, filled circles) \(R^2=0.45, n=12, P=0.02\). In runners, a 94\% (± 1.7 S.E.) of BrdU cells in the granular layer differentiated into neurons (as indicated by double labeling with NeuN) as compared to 86\% (± 3.3) in sedentary mice \[Deviance(1,8)=2.7, P=0.03\]. Volume of the granular layer increased by 18\% (Fig. 2.3C) \([t(22)=2.6, P=0.02]\), and density of blood vessels by 16\% (Fig. 2.3D) \([t(21)=5.6, P<0.0001]\).

Whole hippocampus

Exercise did not significantly change volume (Fig. 2.3C) or vascular density (Fig. 2.3D), but the trend was for a 5\% and 7\% increase in each variable, respectively.

Whole striatum

Exercise did not significantly change volume of the entire striatum (Fig. 2.3C) but the trend was for a 4\% increase. Vascular density significantly increased by 7\% (Fig. 2.3D) \([t(21)=2.5, P=0.02]\).

Experiment 2: Functional analysis of new neurons in high-running B6D2F1/J mice.

In the young adult B6D2F1/J mice (both sexes), wheel running increased neurogenesis 4.5 fold (Fig. 2.3A) \([t(10)=6.8, P<0.0001]\). Level of running was not significantly correlated with number of new neurons among individuals (Fig. 2.3B, open squares). The percentage of BrdU cells double labeled with NeuN was 95\% (±1.0) in runners and 83\% (± 2.8) in sedentary mice \[Deviance(1,10)=7.3, P<0.0001\]. Volume of the granular layer increased by 18\% (Fig. 2.3C) \([t(10)=3.8, P=0.004]\). There was a non-significant trend for density of blood vessels to increase by 7\% (Fig. 2.3D). Effects of sex or interactions between sex and treatment were not significant for any of the variables.

c-Fos induction from wheel running in F1 mice

Running significantly increased the number of c-Fos positive cells in the dentate gyrus by approximately 5-fold (Fig. 2.4E) \([t(10)=3.7, P=0.004]\). Sedentary mice had an average of 950 (± 177 S.E.) c-Fos positive cells per cubic mm dentate gyrus whereas
Runners had 4,533 (± 951) cells. The Pearson’s correlation (r) between level of running within 90 min before euthanasia and number of c-Fos positive cells among the 6 individuals was 0.78. This was marginally not significant due to the small sample size (P = 0.07). However, the correlation was even stronger (r = 0.87) and significant (P=0.03) for distance run the first 60 minutes of the 90 min period (Fig. 2.4E). One individual female mouse happened to run very little within this period (0.1 km/hr) and had very few c-Fos positive cells (753 per cubic mm). All the other animals ran an order of magnitude farther (range from 1.0 to 1.4 km/hr) and numbers of c-Fos cells were also much higher (they ranged from 3,612 per cubic mm to 7,446). This mouse was not a low runner on previous days. In fact, across the entire study, this individual ran the most (an average of 9.5 km/day). There was no evidence that this mouse was sick at the time of sampling, and the mouse ran normal levels on the previous day. Hence, we believe the low running within the 90 minute sampling window on the final day was a chance event for this individual. When this mouse was excluded from the analysis, the correlation between level of running and number of c-Fos cells remained high despite the low sample size and reduced range among the runners (n=5, R2= 0.64). With this low runner removed, the mean level of c-Fos cells per cubic mm in the runner group increased to 5,290 (± 644).

Phenotypic analysis of NeuN-positive cells for co-expression of c-Fos

We examined a total of 2,151 BrdU-positive neurons (BrdU+, new neurons) and 73,722 BrdU-negative neurons (BrdU-, neurons unlabeled with BrdU) in the granule cell layer of the dentate gyrus to determine the proportion expressing c-Fos (Fig. 2.4A-D). The BrdU+ cells were concentrated in the inside layer, adjacent to the hilus where 77% of the 2,151 cells were located. In contrast, the c-Fos cells were evenly distributed from the inside layer (adjacent to the hilus, where 15% of the cells were located) to the outer edge of the granular layer (away from the hilus) where the remaining 85% were located (Fig. 2.4F).

In sedentary mice, the percentage of c-Fos positive neurons was very low, between 0 to 0.2 (± 0.002 S.E.) percent (see Fig 2.4F) and was similar for BrdU+ versus BrdU- neurons and similar for the inside layer versus the outer portions of the granule cell layer. In runners, the percentage of c-Fos cells was higher [Deviance(1,22)=182.9, P<0.0001] ranging from 0.7 (± 0.002) to 3.3 (± 0.008) percent depending on BrdU.
labeling and location in the granule layer (Fig. 2.4F). This was reflected by a significant main effect of BrdU labeling [i.e., BrdU+ versus BrdU-, Deviance(1,22)=4.4, $P=0.04$], a significant main effect of granule cell region [inside layer adjacent to the hilus versus layers away from the hilus, Deviance(1,21)=6.6, $P=0.01$] and a significant interaction between BrdU labeling and region [Deviance(1,20)=9.2, $P=0.002$] in the logistic regression. BrdU+ cells in the outer portions of the granular layer (i.e., not adjacent to the hilus) were 5 times as likely to display c-Fos than BrdU+ cells in the inside layer or as compared to BrdU- cells in either region (Fig. 2.4F). The percentage of c-Fos cells among BrdU+ cells collapsed across region (i.e., considering the entire granule cell layer without separating inside hilus layer from the rest), was 1.4 % ($\pm$ 0.003) as compared to 0.8 % ($\pm$ 0.001) for BrdU- cells throughout the granular layer.

Behavioral Performance

Experiment 1: C57BL/6J males (Fig. 2.5)

Water maze (150 cm diameter, 8.5 cm square platform)

Acquisition. Both runners and sedentary mice learned the water maze as indicated by significantly decreased path length [F(4,88)=6.8, $P<0.0001$] and latency [F(4,88)=11.9, $P<0.0001$] to reach the hidden platform across days (Fig. 2.5). Runners displayed shorter path lengths [F(1,88)=10.9, $P=0.001$] and latency [F(1,88)=4.6, $P=0.04$] as compared to sedentary mice across days. The interaction between exercise treatment and day was not significant. No differences in swim speed were detected between the groups.

Probe test (1 hour post training). One hour after the final acquisition trial, both runners and sedentary animals displayed significantly more time in the target quadrant than any other quadrant (all $P<0.0001$) and on average, animals crossed through the platform location at least 1 time. No significant differences were detected between runners and sedentary mice for duration or number of crosses through the hidden platform.

Rotarod

Both runners and sedentary mice learned the rotarod as indicated by a significant increase in latency to fall off as the trials progressed [F(11,253)=5.6, $P<0.0001$]. Runners performed better than sedentary mice across all trials [F(1,253)=25.2, $P<0.0001$; a difference of approximately 9 seconds]. No interaction between exercise treatment and trial was detected.
Experiment 2: B6D2F1/J mice (Fig. 2.6)

Water maze (100 cm diameter, 8.5 cm square platform)

Acquisition. Both runners and sedentary mice learned the water maze as indicated by significantly decreased path length \[F(4,40)=10.0, P<0.0001\] and latency \[F(4,40)=19.3, P<0.0001\] to reach the hidden platform across days (Fig. 2.6). Runners displayed a steeper learning curve, as reflected by a significant interaction between exercise treatment and day for latency \[F(4,40)=3.5, P=0.02\]. For path length, the interaction was not significant but the trend was in the same direction \[F(4,40)=2.4, P=0.07\]. No main effect of exercise treatment was detected. No differences in swim speed were detected between the groups.

Probe test (24 hours post training). Twenty-four hours after the final acquisition trial, both runners and sedentary animals spent approximately 15 seconds in the target quadrant, the expected time based on random performance. On the other hand, both groups crossed through the location of the hidden platform on average between 4 or 5 times. No significant differences were detected between runners and sedentary mice for duration or number of crosses through the hidden platform.

Visible platform water maze.

Both runners and sedentary mice learned the task as indicated by significantly decreased path length \[F(2,20)=4.9, P=0.02\] and latency \[F(2,20)=7.6, P=0.004\] to reach the visible platform across days. However, no effect of exercise or interaction between exercise treatment and day was observed.

Rotarod.

Both runners and sedentary mice learned the rotarod as indicated by significantly increased latency to fall off as the trials progressed \[F(11,110)=3.2, P<0.001\]. Runners performed similar to sedentary mice. No interaction between exercise treatment and trial was detected.

Discussion

The discovery that exercise increases the growth of new nervous tissue in the adult hippocampus has generated great interest and enthusiasm. If we can understand how an effective natural regenerative system works in the adult mammalian brain, then that holds promise for reverse engineering treatments for regenerative medicine. It is not
surprising therefore, that research in this area has grown rapidly in recent years. This paper adds two new pieces of information to the growing literature. First, the results establish that in young adult male C57BL/6J mice, exercise increases the percentage of blood vessels in the granule layer of the dentate gyrus whereas the change is smaller in the hippocampus and striatum. That suggests a specific role for the dentate gyrus in neuronal response to exercise (Pereira et al., 2007). The second novel piece of information is the discovery that new neurons, 7-8 weeks old, are preferentially recruited into the neuronal c-Fos induction from wheel running. This is important because it provides novel evidence for the functional significance of exercise-induced neurogenesis. It is possible that, in addition to extending plasticity for spatial learning and memory (van Praag et al., 1999a, van Praag et al., 2005, Kee et al., 2007b), new neurons generated from exercise play a role in the function of the hippocampus in aerobic physical activity (Rhodes et al., 2003b).

Vascular density

The 16% increase in vascular density (from exercise) in the granular layer of the dentate gyrus was greater than in the whole hippocampus (where the difference was not significant) suggesting that the changes are specific to the dentate gyrus (Fig. 2.3). The percentage increase in the granular layer was also greater than the increase in the striatum, where previous reports of changes in vascular tissue from exercise have been documented (Ding et al., 2004a, Ding et al., 2004b, Ding et al., 2006c). These results support and extend van Praag (2005) for the granular layer of the dentate gyrus. However, the 7% increase in vascular density observed in the striatum is dramatically different from the 10-fold increase reported in rats (Ding et al., 2004a, Ding et al., 2004b, Ding et al., 2006c). The explanation for this difference is not clear. They examined the dorsolateral striatum whereas we analyzed the whole striatum, so it could reflect regional differences. Also they only counted capillaries less than 25 microns whereas we counted all stained vascular tissue. It also may be due to species (rat versus mouse). On the other hand, a 10-fold change in area covered by blood vessels seems extremely high based on our observations of repeatable individual differences in blood vessel density throughout the brain (e.g., Fig. 2.2A). In our experience, the various immunohistochemical methods available for visualizing vascular tissue (including antibodies against CD-31 or Laminin,
and Lectin stains) can produce inconsistent results. Therefore, it is possible that the difference is due to incomplete staining in (Ding et al., 2004a, Ding et al., 2004b, Ding et al., 2006c) where Laminin was used (e.g., see figure 3B in Ding et al., 2004a).

The result that angiogenesis accompanies neurogenesis in the granular layer is not surprising because new tissue requires metabolic support (i.e., oxygen, nutrients, waste elimination). However, given that the new cells may increase the entire volume of the granular layer, it is not clear why the new vessels do not simply expand in proportion to the added nervous tissue producing more total vascular tissue without changing density. One possibility is that increased blood flow to this region (Pereira et al., 2007) associated with greater neuronal activation (Czurko et al., 1999, Rhodes et al., 2003a), resulted in greater perfusion of both old and new neurons.

**Recruitment of new neurons in the c-Fos induction from running.**

In Experiment 2, we entertained the hypothesis that new neurons from running might play a role in the c-Fos induction from the wheel running itself (Rhodes et al., 2003b). We reasoned that if wheel running activated neurons in the granular layer (Rhodes et al., 2003a), and the neuronal activity contributed to the signaling for the generation of new neurons in that region, then the new neurons might be recruited into the neuronal response once they are mature. Results confirmed this hypothesis. New (BrdU labeled) neurons (7-8 weeks old) were significantly more likely to be recruited into the neuronal c-Fos response to running as compared to the population of unlabeled neurons (a combination of older neurons and new neurons unlabeled with BrdU) (Fig. 2.4F). Importantly, this difference was only for new neurons that had migrated into the granule cell layer away from where they were born (i.e., the inside layer adjacent to the hilus) (van Praag et al., 2002). These results suggest either that the new neurons located in the middle of the granule cell layer were more mature (i.e., labeled for BrdU earlier during the 10 day treatment) and hence more integrated into the circuit, or that only a subset of new neurons migrated into the interior of the granule cell layer, and those were the ones involved in running. Future research is needed to explore different age classes of new neurons to determine when maximum recruitment occurs into the c-Fos response to running.
The discovery that new neurons generated from exercise are preferentially recruited into neural activation from running is important because it provides new evidence for the functional significance of exercise-induced neurogenesis. Previous studies have suggested that new neurons from exercise play a role in extending plasticity for spatial learning and memory (van Praag et al., 1999a, Kee et al., 2007b, Clark et al., 2008). Our data suggests it is also possible that new neurons generated from exercise play a role in the function of the dentate gyrus in the wheel running behavior itself. It is not clear why neuronal activity in the hippocampus is closely correlated with wheel running speed (Fig. 2.4E) (Czurko et al., 1999, Rhodes et al., 2003a) or why new neurons are needed to support that activation. It is possible that the new neurons replace older, dying or dead neurons, or that they are more plastic and therefore more likely to take on whatever function the hippocampus is playing (whether wheel running or solving a water maze).

Behavioral performance

Previous studies have established that C57BL/6J genotype displays pro-cognitive effects of exercise on the Morris water maze, although the effects have always been small and depend on the parameters used for the maze (van Praag et al., 1999a, van Praag et al., 2005, Clark et al., 2008). In this study, runners displayed significantly shorter path lengths and latencies to the hidden platform across days during acquisition, but they did not differ during the probe trial (see Fig. 2.5). In a previous study using C57BL/6J, we used a smaller maze (70 cm diameter with the same size platform as here) but similar parameters for the exercise treatment. In that study, differences between runners and sedentary mice were smaller for acquisition than reported here, but larger and significant for the probe trial (Clark et al., 2008). The larger maze used in this study (150 cm diameter) may have been more difficult for the animals. Evidence for this is that the animals appeared to remember the location of the target quadrant during the probe trial but they were unable to pinpoint the exact location of the hidden platform, as indicated by relatively few crossings (Fig. 2.5).

Although we observed a strong main effect in the C57BL/6J model, with runners performing better than sedentary mice across days during acquisition, we did not find a significant interaction between exercise treatment and day, implying that the slope of the
learning curves were similar (i.e., the runner group had a constant advantage). On the other hand, on the very first trial (data not shown), none of the mice made it to the platform by themselves within the 1 minute period, and average latency and path length to the platform was the same between the groups. The runner advantage began to emerge by the second trial, and therefore differences in the steepness of the learning curves may have been obscured by averaging the trials together on the first day. Taken together, results suggest that the exercise treatment used in this study resulted in benefits in behavioral performance on the water maze in the C57BL/6J genotype, and that the dimensions of the water maze can influence whether effects are seen during acquisition or in the probe test.

The rotarod data show that exercise can also improve behavioral performance on a task NOT hypothesized to involve the hippocampus in the C57BL/6J model. This is consistent with the growing knowledge that the benefits of exercise are broad and not limited to the hippocampus. The neuroanatomical variables that changed from exercise (Fig. 2.3) occurred in parallel with gain in performance on the water maze and the rotarod (Fig. 2.5). This makes it more difficult to identify causal connections between specific physiological responses such as neurogenesis and specific cognitive outcomes such as spatial memory enhancement. One way to tackle this problem is to directly manipulate the hypothesized substrates such as neurogenesis to determine whether the substrates are required for specific pro-cognitive benefits of exercise (Clark et al., 2008).

The behavioral results for the F1 genotype were more complicated. The water maze was smaller (100 cm diameter), and the F1 sedentary mice started out performing slightly better than the runners on day 1. However, by the end of the 5 days of training, runners performed better than sedentary. Hence, over the 5 days of training, runners displayed a steeper learning curve than sedentary mice. The probe test occurred 24 hours after the final training trial. Therefore, it is not clear whether the poor performance demonstrated by all the mice, as indicated by equal time spent in all quadrants, was a reflection of the mice forgetting the location of the platform or adopting an alternative strategy of random searching after realizing that the platform was gone. The large number of crossings is consistent with the latter explanation.
The visible platform data (Fig. 2.6) are difficult to interpret because the mice performed well on the first day (labeled day 7 on the graph in Fig. 2.6), leaving little room for improvement (i.e., ceiling effect). However, the statistically insignificant trend was for runners to perform better than sedentary mice which would suggest that the steeper slope on the hidden version may have reflected a general performance gain rather than one specific for spatial ability. On the other hand runners did not differ from sedentary mice for swim speed or rotarod performance, suggesting that their motor performance was similar. Taken together, in the F1 genotype, runners may have learned the water maze faster than sedentary mice, but more work is required to evaluate the extent to which the benefit is related to spatial learning and memory as opposed to other variables that can influence performance on these tasks.

**Strain comparisons**

The purpose for using two different strains in this study was NOT to characterize genetic differences. C57BL/6J was chosen for experiment 1 because pro-cognitive effects of exercise have already been established for this strain (van Praag et al., 1999a, van Praag et al., 2005, Clark et al., 2008). The B6D2F1/J strain was chosen for experiment 2 because they run more than C57BL/6J (Fig. 2.1) and we reasoned they would be more likely to show overlap between numbers of new neurons and c-Fos positive cells in the granular layer because they would generate more of each. Because of these different experimental priorities, the two strains were studied in two separate experiments with slightly different parameters (see Materials and Methods). Therefore, strong conclusions about genetic differences are not possible because the environment too, was different. More than 2 genotypes are required anyway to draw statistical inferences about genetic correlations between physiology and behavior (Garland and Adolph, 1994). That being said, a few observations are worth noting. First, the F1 mice (both sexes) ran much more than the male C57BL/6J mice, as expected (Fig. 2.1). Also, level of running was correlated with number of new neurons among C57BL/6J runners, but not F1 (Fig. 2.3B). This is consistent with results of a selective breeding experiment where high running lines (that ran approximately 12 km/day) showed no correlation between amount of running and number of new neurons whereas the control lines, that ran at moderate levels (approximately 4 km/day), showed a strong correlation (Rhodes et
One explanation for this result is that high-running mice reach the physiological limit, ceiling, or capacity for generating new neurons from physical activity (Rhodes et al., 2003b).

Another interesting difference was that exercise improved performance on the rotarod in C57BL/6J, whereas exercise had no effect in the F1 (Fig. 2.5 versus Fig. 2.6). High baseline performance in the sedentary group may have obscured performance gains in the F1 (i.e., ceiling effect). Results might have been different had we used different parameters for the rotarod (e.g., increased rate of acceleration, diameter of the dowel, etc.) (Rustay et al., 2003).

Summary

There is growing evidence that the dentate gyrus is a major locus for phenotypic change induced from exercise in the brain (Christie et al., 2008, van Praag, 2008). Here we extend this recent literature, by showing that density of blood vessels significantly increases in the granular layer of the dentate gyrus in the C57BL/6J mouse model. This increase in vasculature may support neurogenesis, neural activity associated with exercise, or as an inevitable consequence of increased blood flow to the region (Pereira et al., 2007). We also demonstrated that new neurons of 7-8 week age class are preferentially recruited into the neuronal response to wheel running in the B6D2F1/J high-running mouse model. This is important because it provides new evidence for the functional significance of exercise-induced neurogenesis. In addition to extending plasticity for spatial learning and memory, new neurons generated from exercise may play a role in the neuronal activation of the hippocampus associated with aerobic physical activity.
Figure 2.1. Wheel running over the course of the study. Distance run (km/day) (± S.E.) shown separately for C57BL/6J (open symbol; n=12 males) and B6D2F1/J mice (closed symbol; n=3 males and 3 females). The first 10 days mice received daily injections of 50 mg/kg BrdU to label dividing cells. The last 10 days mice were tested on water maze then rotarod, during the light phase of the light/dark cycle when levels of wheel running are negligible.
Figure 2.2. Blood vessels in the hippocampus.  A) A representative section stained with an antibody against collagen IV with diaminobenzidine as the chromogen, combined with a light Nissl stain to visualize blood vessels in and around the dentate gyrus (100X total magnification).  B) The same section at 200X total magnification with grid overlaid and region outlined to illustrate the method for estimating area fraction covered by vessels in the granular layer. Twelve or more such pictures per animal were analyzed to count the number of vertices intersecting with vascular tissue within defined brain regions.
Figure 2.3. Neuroanatomical changes induced from exercise. A) Average number of new neurons per cubic mm (±SE) shown separately for sedentary versus runners in the C57BL/6J and the B6D2F1/J genotypes. Open bars are sedentary, filled bars are the runners. B) Number of new neurons per cubic mm in individual runners plotted against the average amount they ran over the course of the study in km/day. Filled circles are C57BL/6J and open boxes are B6D2F1/J. C) Average volume (±SE) of the granular layer of the dentate gyrus in C57BL/6J and B6D2F1/J, the whole hippocampus in C57BL/6J, and the whole striatum in C57BL/6J shown separately for runners versus sedentary mice. D) The same as C except for blood vessel area fraction. * indicates p<0.05, **p<0.01, *** p<0.001.
Figure 2.4. New neurons are recruited into the c-Fos induction from wheel running in B6D2F1/J mice. A-C) Representative sections from B6D2F1/J runners triple labeled for BrdU (green), NeuN (blue), and c-Fos (red) sampled 2 hours after the lights shut off in the animal room. D) The area within the white box in panel C, zoomed in, showing a cell
that was triple labeled, indicating an episode where a new neuron displayed c-Fos in response to running. E) Number of c-Fos positive cells shown for each individual sedentary animal (open triangles) or runner (filled circles) plotted against the amount they ran within a 60 minute window starting 90 minutes before the animals were removed from their wheels and euthanized. Note one runner ran very little (approximately 0.1 km/hr) within this window relative to the others. The sedentary animals were assigned a value of zero for distance run. F) The percentage of neurons (i.e., NeuN positive cells) in the granular layer displaying c-Fos shown separately for Sedentary (far right) versus Runner groups (left), BrdU positive (BrdU+, new neurons) versus BrdU negative (BrdU-, unlabeled with BrdU) neuronal populations, and for cells lining the inside of the granule cell layer adjacent to the hilus (open bars labeled “Hilus layer”) versus cells in outer layers (gray bars, labeled “Middle GCL”). Standard error bars estimated from logistic regression are shown.
Figure 2.5. Effects of exercise on behavioral performance in C57BL/6J mice. Runners (filled symbols, filled bars) are shown separately from the sedentary group (open symbols, open bars). For water maze (150 cm diameter), acquisition, mean path length (m) and latency (sec) across days are shown. For the water maze probe trial (1 hour after the last training trial), mean duration in the target quadrant (sec) and number of crossings through the platform location are shown. For rotarod, mean latency (sec) to fall on each of 12 trials (4 trials per day for three days) are shown. Standard error bars are shown throughout.
Figure 2.6. Effects of exercise on behavioral performance in B6D2F1/J mice. Runners (filled symbols, filled bars) are shown separately from the sedentary group (open symbols, open bars). For water maze (100 cm diameter), the hidden platform acquisition (days 1-5) and visible platform acquisition (days 7-9) are shown as mean path length (m) and latency (sec) across the days. For the probe test (on day 6, 24 hours after the last hidden platform training trial), mean duration in the target quadrant (sec) and number of crossings through the platform location are shown. For rotarod, mean latency (sec) to fall on each of 12 trials (4 trials per day for three days) are shown. Standard error bars are shown throughout.
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CHAPTER 3

Induction of c-Fos, Zif268, and Arc from acute bouts of voluntary wheel running in new and pre-existing adult mouse hippocampal granule neurons.

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Abstract

The functional significance of newly formed granule neurons in the adult mammalian hippocampus remains a mystery. Recently, it was demonstrated that wheel running increases new neuron survival and c-Fos expression in new and pre-existing granule cells in an activity-dependent manner. It is currently unknown whether other immediate early genes (IEGs) become expressed in granule neurons from running. Further, it is unknown whether locomotor activity in home cages without wheels can influence neurogenesis and IEG expression similar to running. The purpose of this study was three fold: 1) to determine if Arc and Zif268 expression are also induced from wheel running in both pre-existing and newly formed neurons 2) to determine if neurogenesis and IEG induction is related to horizontal distance traveled in home cages without wheels and 3) to determine whether IEG induction is related to acute bouts of running or chronic effects. Adult C57BL/6J female mice were placed in cages with or without running wheels for 31 days. The first 10 days, mice received daily injections of 5-Bromo-2'-deoxyuridine (BrdU) to label dividing cells. On day 31, running and non-running animals were euthanized either 2 hours after peak activity, or during a period of relative inactivity. Immunohistochemistry was performed on hippocampal sections with antibodies against BrdU, mature neuron marker NeuN, c-Fos, Arc, and Zif268. Results demonstrate that Arc, Zif268, and c-Fos are induced from wheel running but not movement in cages without wheels. All IEGs were expressed in new neurons from running. Further, IEGs were induced acutely by running, as increased expression did not continue into the light cycle, a period of relative inactivity. The results suggest that robust movements, like running, are necessary to stimulate IEG expression and neurogenesis. Moreover, results suggest new neurons from running may be processing information about running behavior itself.
Introduction

Aerobic running, either voluntary or forced, stimulates the formation of granule neurons in the adult rodent hippocampus (van Praag et al., 1999b, Uda et al., 2006). The number of new neurons generated is strongly related to distance run in mice housed with exercise wheels (Rhodes et al., 2003b, Bednarczyk et al., 2009, Clark et al., 2009, Clark et al., 2011). Moreover, exercise-induced neurogenesis can increase the volume of the entire granule cell layer by adding to the total number of granule neurons (Rhodes et al., 2003b, Clark et al., 2008). These discoveries have drawn much interest, as they provide evidence that the generation of new nervous tissue in the adult mammalian brain is possible and can be stimulated naturally by physical activity. However, very little is known about the function of new neurons from wheel running (Clark et al., 2008, Clark et al., 2009). Moreover, how new neurons function during hippocampal-dependent tasks remains a hotly debated topic (Kempermann, 2002, Kempermann et al., 2004, Schinder and Gage, 2004, van Praag, 2009, Deng et al.).

One way to assess the function of new neurons in the adult hippocampus is to determine whether they become selectively activated while performing a hippocampal-dependent task. Immediate early gene (IEG) expression has been widely accepted as a marker for neuron activation, although the physiological mechanisms underlying their expression are not well understood (Guzowski et al., 2005). Recent studies have used the expression of IEGs such as c-Fos, Zif268, and Arc to demonstrate that new granule neurons become activated during performance on hippocampal-dependent cognitive tasks (Jessberger and Kempermann, 2003, Ramirez-Amaya et al., 2006, Kee et al., 2007, Tashiro et al., 2007, Snyder et al., 2009c, Trouche et al., 2009). It has been hypothesized that this activation indicates a role for new neurons in hippocampal-dependent learning and memory retrieval.

The assessment that new neurons are activated during hippocampal-dependent learning or memory retrieval can be problematic as many recent studies have not properly controlled for the effects of acute bouts of locomotor activity required to perform the cognitive task on inducing IEGs. These studies typically compared IEG expression of animals performing a behavioral task to animals that remained housed in standard laboratory cages, or did not equate the relationship between IEG expression and distance.
traveled while performing the task (Jessberger and Kempermann, 2003, Ramirez-Amaya et al., 2006, Kee et al., 2007, Tashiro et al., 2007, Trouche et al., 2009).

There is reason to speculate that IEG expression in the granule cells may be induced by the physical activity necessary to complete hippocampal-dependent task. One not widely known or underappreciated feature of hippocampus granule neurons is that they are quantitatively activated by acute bouts of voluntary movement. For example, wheel running increases c-Fos expression in the granule cell layer which is strongly related \((r > 0.88)\) to the distance the animal traveled 90-120 minutes before euthanasia (Rhodes et al., 2003a, Clark et al., 2009, Clark et al. 2010). The acute c-Fos response from running does not habituate and the induction continues at a similar level even after 50 days of continuous access to running wheels (Lee et al., 2003, Clark et al. 2010). Our lab has recently reported that new granule neurons preferentially display c-Fos in response to acute bouts of running as compared to pre-existing granule neurons (Clark et al., 2009). While it is well established that c-Fos is up-regulated from running in the dentate gyrus, to the best of our knowledge, whether other IEGs become up-regulated from acute bouts of running in new and pre-existing granule neurons is unknown. It is also unknown if IEG expression in the granule cell layer can be induced in an activity dependent manner during home cage activity, as opposed to more robust repetitive movements specific to running.

The purpose of this study is three-fold. The first objective of this study is to determine which IEGs are most influenced by acute locomotor activity in new and pre-existing granule neurons. Arc and Zif268 were chosen because these IEGs, as well as c-Fos, are commonly used as markers of new neuron activation in association with hippocampal-dependent learning and memory task performance (Jessberger and Kempermann, 2003, Ramirez-Amaya et al., 2006, Kee et al., 2007, Tashiro et al., 2007, Snyder et al., 2009c, Trouche et al., 2009). Further, these IEGs are also well characterized for their expression following learning tasks and the induction of long-term potentiation (as reviewed in Abraham et al., 1991, Dragunow, 1996, Knapska and Kaczmarek, 2004, Bramham et al., 2008). Occasionally, these IEGs can be regulated differently by the same stimulus (Douglas et al., 1988, Cole et al., 1989, Dragunow et al., 1989, Wisden et al., 1990, Kaczmarek et al., 1999, Guzowski et al., 2001). Identifying
IEGs that can distinguish between neuronal activation related to cognitive function as opposed to locomotor activity is crucial for understanding the functional contribution of new neurons in cognition.

The second objective of this study was to determine whether IEG activation and neurogenesis is related to distance traveled in home cages without running wheels. One possibility is that only traveling distances at fast speeds, sufficient to tax aerobic capacity and activate the stress response, can induce IEG expression in the granule cell layer. If this is the case, then normal locomotion in home cages would not be enough to stimulate IEG expression or new neuron formation in an activity-dependent manner. The outcome of this experiment is important for determining whether acute bouts of movement, similar to those necessary to complete a small number of trials on less physically taxing cognitive tests can induce IEGs, or whether robust repetitive movements are necessary to induce IEG expression in granule cells.

The third objective of this study was to determine whether an elevation of IEGs from running is a result of a long-term increase in basal IEG expression, or the reflection of acutely induced cellular activity. IEGs have been most commonly used as markers for acute neural activity. However, IEGs have a baseline expression in the brain that varies between regions and is maintained by ongoing synaptic or hormonal signaling (Beckmann and Wilce, 1997, Herdegen and Leah, 1998, Lee et al., 1998, Shirayama et al., 1999, Guzowski et al., 2001). Fluctuations from basal IEG concentrations can be readily detected following application or removal of a stimulus (Lee et al., 1998, McGahan et al., 1998, French et al., 2001). The hippocampal granule cell layer is a unique brain area in that basal levels of c-Fos, Zif268, and Arc are all low (Gass et al., 1992, French et al., 2001). However, a possibility exists that running may induce a long-term upward shift in the basal IEG protein expression.

The results of this study provide insight into the function of new neurons generated from running. Further, results provide useful information for future studies using IEGs as markers of neuronal activation in cognitive tests assessing the function of new neurons.
Materials and Methods

Animals and husbandry

Cohorts of female C57BL/6J mice arrived at the Beckman Institute Animal facility from The Jackson Laboratory at 5 weeks of age. Females were used because they run more than males (Clark et al., 2011). Upon arrival, mice were housed four per standard polycarbonate shoebox cages with corncob bedding, 7097 ¼ (Harlan Teklad, Madison, Wisconsin, USA) for 2 weeks. The mice were then individually housed either in standard shoe box cages, custom home cages (without wheels) used for video tracking, or cages with wheels as described below. Rooms were controlled for temperature (21 °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.

Dimensions of running wheel cages were 36x20x14 cm (LxWxH) with a 23 cm diameter wheel mounted in the cage top (Respironics, Bend, OR). Wheel rotations were monitored continuously in 1 min increments throughout the experiments via magnetic switches interfaced to a computer. Dimensions of home cages without wheels for video tracking were 34x18x16 cm (LxWxH). Video tracking cages were constructed out of clear plastic with food and water access mounted on the side and clear plastic lids. Dimensions of standard polycarbonate shoebox cages without wheels were 29x19x13 cm (LxWxH).

Experimental Design

Experiment 1: IEG induction from wheel running in new and pre-existing granule neurons (n=16)

The primary objective of this experiment was to determine whether Arc, Zif268, and c-Fos become up-regulated from wheel running in new and pre-existing granule neurons. The secondary objective of this experiment was to determine whether 21-31 day old new neurons show a greater IEG expression from running as compared to pre-existing neurons.
Mice (7 weeks old) were placed individually in cages either without (n = 6; Sedentary) or with running wheels (n = 10 Runners) for 31 days. The first 10 days all mice received daily injections of 50 mg/kg 5-Bromo-2’-deoxyuridine (BrdU) to label dividing cells. Note that mice were deliberately not housed in cages with locked wheels because mice climb in locked wheels and we wanted to keep physical activity to a minimum in the sedentary group. On day 32, mice were euthanized 2 hours after the height of their active period, approximately 3-5 hours after the lights shut off in the animal room.

Experiment 2: Neurogenesis and IEG induction in home cages without wheels (n=8)

The objective was to determine if IEG activation and new neuron formation is related to distance moved in home cages without running wheels.

Mice (7 weeks old) were individually housed in custom-made home cages for video tracking for 31 days. Starting on day 3, all mice received daily injections of 50 mg/kg BrdU for 10 days, to label dividing cells. Horizontal distance traveled per day in the home cages was recorded for each sedentary mouse by overhead video cameras and analyzed using TopScan 2.0 (Clever Sys, Vienna, VA, USA) video tracking software (Zombeck et al., 2008). On day 32, mice were euthanized 2 hrs after the height of their most active period, approximately 3-5 hrs after the lights shut off in the animal room.

Experiment 3: IEG induction during the inactive light phase (n=24)

The objective of this experiment was to determine whether an elevation of IEGs from running reflects a long-term increase in basal IEG expression or acute cellular activity. If IEG expression in the granule cell layer from running is a result of a long-term increase in basal expression, then we reasoned that IEG expression will remain elevated during a period of relative inactivity following several hours of running. Number of wheel revolutions were monitored during the light cycle, a period during which running is minimal. If IEG expression decreases in correlation with decreased distance traveled or is no different from sedentary mice, this would constitute evidence that IEG induction from running is a reflection of acute cellular activity. On the other hand, if IEG induction remains elevated even though running has decreased, this would indicate that running increases basal IEG expression in granule cells.
Mice (7 weeks old) were individually housed in cages with (n = 18; Runners) or without (n=6; sedentary) running wheels for 31 days. The first 10 days all mice received daily injections of 50 mg/kg BrdU to label dividing cells. On day 32, runner mice were euthanized in three groups (n = 6 per group) at 3, 6, or 9 hours after the onset of the light cycle in the animal rooms (a period of relative inactivity for mice). In addition, 2 sedentary mice were euthanized at each respective time point.

**Immunohistochemistry**

Following Clark et al. (2008), mice were anesthetized with 150 mg/kg sodium pentobarbital (ip) and then perfused transcardially with 4% paraformaldehyde in a phosphate buffer solution (PBS). Brains were post-fixed overnight, and transferred to 30% sucrose in PBS. Brains were sectioned using a cryostat into 40 micron coronal sections and stored in tissue cryoprotectant at -20°C. Separate 1-in-8 series of these sections (i.e., series of sections throughout the rostro-caudal extent of the brain with 320 micron increments separating each section) were stained in each of the following ways.

1) BrdU-DAB. Purpose: To detect BrdU-positive (newly divided) cells in the dentate gyrus. Free floating sections were washed in TRIS-buffered solution (TBS) and then treated with 0.6% hydrogen peroxide. To denature DNA, sections were treated with 50% de-ionized formamide, 10% 20XSCC buffer, 2N hydrochloric acid, and 0.1 M Boric acid. Sections were then treated with a solution of 0.3% Triton-X and 3% goat serum in TBS (TBS-X plus), and then incubated in primary antibody against BrdU made in rat (Accurate, Westbury, NY) at a dilution of 1:100 in TBS-X plus for 72 hrs 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) and stained using a diaminobenzidine kit (Sigma, St. Louis, MO).

2) Arc, c-Fos, Zif268 -DAB. Purpose: To detect the presence of IEGs in the granule cell layer. Free floating sections were washed in Phosphate-buffered solution (PBS) and then treated with 0.6% hydrogen peroxide. Sections were then treated with a solution of 0.2% Triton-X and 5% goat serum in PBS (PBS-X plus) for 1 hour, and then incubated in primary antibody against c-Fos (Calbiochem, San Diego, CA), Arc (SySy, Goettingen, Germany), or Zif268 (Santa Cruz Biotech, Santa Cruz, CA) made in rabbit at a dilution of
1:18,000, 1:10,000, or 12:000 respectively in PBS-X plus for 48 hrs at 4 °C. Sections were then washed in PBS, treated with PBS-X plus for 60 min and then incubated in secondary antibody against rabbit made in goat at 1:250 in PBS-X plus for 90 min at room temperature. Sections were then treated using the ABC system (Vector, Burlingame, CA) and stained using a diaminobenzidine kit (Sigma, St. Louis, MO).

3) Triple-fluorescent label. To determine the proportion of BrdU+ and BrdU-negative (BrdU-) neurons in the dentate gyrus that expressed an IEG. The procedure for BrdU-DAB was repeated except for the following. A cocktail was used for the primary antibody step that included rat anti-BrdU (1:100; Accurate, Westbury, NY), mouse anti-NeuN (1:50; Chemicon, Billerica, MA), and either rabbit anti-c-Fos (1:7000; Calbiochem, San Diego, CA), anti-Arc (1:1000; SySy, Goettingen, Germany), or anti-Zif268 (1:2000; Santa Cruz Biotech, Santa Cruz, CA). Secondary antibodies made in goat were conjugated with fluorescent markers (Cy2 anti-rabbit, Cy3 anti-rat, Cy5-anti mouse; Jackson ImmunoResearch, West Grove, PA) at dilution 1:200 and also delivered as a cocktail.

Image analysis

1) BrdU-DAB, Arc-DAB, c-Fos-DAB, and Zif268-DAB. Following Clark et al., 2008, the entire granule layer (bilateral), represented in the 1-in-8 series was photographed by systematically advancing the field of view of the Zeiss brightfield light microscope, and taking multiple photographs, via camera interfaced to computer, under 10X (total 100X) magnification. Positively labeled cells in these photographs were counted to generate unbiased estimates of total number of labeled cells (BrdU, Arc, c-Fos, or Zif268). In 6 runners, IEG cells counts were conducted separately for the supra- or infra-pyramidal blades of the granule cell layer for comparison of counts between locations. Positively labeled cells that were predicted to be in the top plane of the sections were not included in cell count estimates. In addition, the total volume of the dentate gyrus represented in the series was measured so that the IEG counts could be expressed per cubic micrometer dentate gyrus sampled. Area per section was obtained by outlining the entire bilateral granule cell layer. Volume was obtained by multiplying average area per section by the number of sections and then by the space between sections for each animal.
2) Triple label. Following Clark et al. (2009), a confocal Leica SP2 laser scanning confocal microscope (using a 40X oil HCX PL APO C5 objective with 1.25 numerical aperture, pinhole size 81.35 μm, 1-Airy Unit) was used to determine the proportion of BrdU cells (BrdU+) that differentiated into neurons (NeuN+) and to determine the proportion of neurons (BrdU+/NeuN+, or BrdU-/NeuN+) displaying an IEG (Arc, c-Fos, or Zif268). Each image sequentially captured a part of the dentate gyrus on a single plane (randomly selected on the z-axis) that was just out of view from previous image. This sequential imaging was done until the entire medial to lateral extent of the each bi-lateral granule cell layer was captured. The bi-lateral granule cell layer was imaged in 3 - 4 hippocampal sections (spanning the rostral, medial, and caudal extent) of 3 - 4 sedentary and farthest running mice. All the new (BrdU+/NeuN+) and pre-existing (BrdU-/NeuN+) granular neurons, as well as the number of neurons co-labeled with an IEG (BrdU-/NeuN+/ IEG+, or BrdU+/NeuN+/ IEG+) in that plane were counted for each image.

IEG-expressing new neurons were also analyzed for the location within the granule cell layer. The number of IEG expressing new neurons that shared a border directly adjacent to the hilus of the granule cell layer was compared with the proportion of IEG expressing new neurons that had migrated into the granule cell layer (as defined by not having a border adjacent to the hilus). This analysis was conducted to determine whether the probability of IEG expression in new neurons differs depending on the location of the new neurons within the granule cell layer.

Statistical Analysis

Data were analyzed using SAS, or R statistical software. In all analyses, P < 0.05 was considered statistically significant.

The proportion of BrdU labeled cells in the granule cell layer that also expressed NeuN was compared in sedentary and runner conditions by logistic regression. To determine whether new neurons are more likely to display an IEG from running, the proportions of NeuN+/BrdU- and NeuN+/BrdU+ neurons expressing an IEG (Arc, c-Fos, or Zif268) were also compared by logistic regression. For these analyses, the deviance is reported in place of an F statistic.
The number of IEG cells per mm³ and total number of new neurons in the granule cell layer was compared between running and sedentary animals using a two sample T-test assuming equal variance. Pearson’s correlations between amount of running (e.g., km/hr or km/day) and number of IEG positive cells or number of new neurons were analyzed using simple linear regression.

In experiment 3, the sedentary average was obtained by collapsing data across pairs of mice sampled 3, 6, or 9 hrs after the onset of the light cycle. The number of IEG cells per mm³ was compared between sedentary and runners sampled 3, 6, or 9 hrs after lights on using a 1-way ANOVA. T-tests with Tukey correction were used for pair-wise group comparisons on statistically significant ANOVAs.

Results

Experiment 1: IEG induction from wheel running in new and pre-existing granule neurons

Wheel Running

Inspection of Figure 3.1A shows that wheel running distance increased steadily for the first 19 days and thereafter maintained a plateau. Average amount of wheel running over the entire experiment was 6.1 km/day (± 0.29 S.E.).

Neurogenesis from wheel running

Wheel running increased neurogenesis approximately 2-fold (Fig. 3.2A & B) \([t(14)=3.2, P<0.006]\). Average daily running distance was significantly correlated \((r = 0.75)\) with number of new neurons among individuals (Fig. 3.2C) \([p = 0.012]\). The percentage of BrdU cells double labeled with NeuN was 94% (±1.0) in runners and 85% (± 1.8) in sedentary mice \([\text{Deviance}=8.6, P=0.003]\). Running caused the volume of the granule cell layer to increase by 13% \([t(14) = 3.6, P=0.002]\).

IEG induction from wheel running

\(c-Fos\)

Running caused approximately a 5-fold increase in the number of c-Fos positive cells in the dentate gyrus (Fig. 3.2D & E) \([t(14)=4.3, P=0.0007]\). Previous literature has established that c-Fos protein is present at 30 mins and peaks at approximately 2 hrs post cell activation then sharply diminishes (Zangenehpour and Chaudhuri, 2002). The Pearson’s correlation \((r)\) between level of running accumulated within 2 hrs before
euthanasia and number of c-Fos positive cells among the 10 individuals was 0.89 (Fig. 3.2F) \([p = 0.0006]\). See Figure 3.3 for a representative plot of running levels at hourly intervals before euthanasia for a representative low, medium and high running mouse. c-Fos expression did not differ between the supra- and infra-pyramidal blades.

**Zif268**

Running caused approximately a 3-fold increase in the number of Zif268 positive cells in the dentate gyrus (Fig. 3.2G & H) \([t(14)=5.7, P<0.0001]\). Previous literature has established that Zif268 protein peaks at approximately 2 hrs following cell activation, but displays a more broad bell shaped expression curve than c-Fos (Richardson et al., 1992, Zangenehpour and Chaudhuri, 2002). The Pearson’s correlation \((r)\) between level of running 2 hrs before euthanasia and number of Zif268 positive cells among the 10 individuals was 0.65 \([p = 0.04]\). However, the correlation was even stronger \((r = 0.79)\) for distance run 4 hours preceding euthanasia (Fig. 3.2I) \([P=0.006]\). Given the large range in Zif268 cell stain intensity (light grey to dark black cells) as compared to c-Fos and Arc, as well as the broad expression curve, it is likely we were able to detect protein expression over a longer period of time. Zif268 expression did not differ between the supra- and infra-pyramidal blades.

**Arc**

Running caused approximately a 3-fold increase in the number of Arc positive cells in the dentate gyrus (Fig. 3.2J & K) \([t(10)=3.0, P=0.009]\). Previous literature has established that Arc protein peaks at approximately 30 min after cellular activation, but remains elevated for at least 8 hours in the granule cell layer (Ramirez-Amaya et al., 2005). Thus, Arc expressed in the granule cell layer may likely represent an accumulation of the previous 8 hours of running. Our data supported this finding, as the relationship between distances traveled before euthanasia and Arc induction continued to improve over time, before peaking at 8 hours. The Pearson’s correlation \((r)\) between level of running 2 hours before euthanasia and number of Arc positive cells was 0.63 \([P < 0.05]\). However, the Pearson’s correlation \((r)\) between level of running 8 hours before euthanasia and number of Arc positive cells among the 10 individuals was 0.90 (Fig. 3.2L) \([P=0.0004]\). Arc expression did not differ between the supra- and infra-pyramidal blades.
Running-induced IEG expression in new neurons

Proportions of BrdU+ and BrdU- neurons expressing c-Fos (Figs. 3.4A & B)

In sedentary mice, a total of 25,420 neurons (NeuN+) unlabeled with BrdU (BrdU-) and 343 new neurons labeled with BrdU (BrdU+) were analyzed for the expression of c-Fos. In runners, a total of 21,117 BrdU- and 666 BrdU+ neurons were analyzed for the expression of c-Fos. The number of neurons expressing c-Fos was greater in the runners as compared to sedentary animals [Deviance=97.6, \( P < 0.0001 \)]. In sedentary mice, the percentage of BrdU- and BrdU+ neurons expressing c-Fos was low and not statistically different from each other. In BrdU- neurons, the percentage of c-Fos was 0.5% ± 0.04 S.E., and in BrdU+ neurons the percentage was also 0.5% ± 0.40 S.E. In runners, the percentage of neurons expressing c-Fos was greater in BrdU+ neurons than BrdU- neurons [Deviance=17.4, \( P < 0.0001 \)]. In BrdU+ neurons the percentage was 3.6% (± 0.72 S.E.) whereas in BrdU- neurons the percentage was 1.3% (± 0.08 S.E.).

Proportions of BrdU+ and BrdU- neurons expressing Zif268 (Figs. 3.4C & D)

In sedentary mice, a total of 15,674 BrdU- and 178 BrdU+ neurons were analyzed for the expression of Zif268. In runners, a total of 11,332 BrdU- and 263 BrdU+ neurons were analyzed for the expression of Zif268. The basal and running-induced Zif268 levels in the dentate gyrus was noticeably greater than c-Fos in the granule cells (see Fig. 3.2D-I). This resulted in a greater overall proportion of neurons expressing Zif268 than c-Fos [Deviance=168.5, \( P < 0.0001 \)]. The percentage of neurons expressing Zif268 was greater in runners than sedentary animals [Deviance=167.9, p<0.0001]. The percentage of BrdU- neurons expressing Zif268 was approximately 1.1% (± 0.08 S.E.) and 3.3% (± 0.17 S.E.) in sedentary and runners, respectively. The percentage of BrdU+ neurons expressing Zif268 was approximately double that of BrdU- neurons for both sedentary at 2.3% (± 1.11 S.E.) and runners at 6.8% (± 1.56 S.E.) [Deviance=13.9, \( P = 0.0002 \)], however post hoc analysis revealed this difference only reached significance for the runners [Deviance= 7.8, \( P = 0.005 \)]. A qualitative difference in the intensity of the Zif268 stain was observed between sedentary and runners under the confocal microscope. Zif268 fluoresced noticeably dimmer in sedentary mice.

Proportions of BrdU+ and BrdU- neurons expressing Arc (Figs. 3.4E & F)
A total of 30,769 BrdU- and 191 BrdU+ neurons were analyzed for the expression of Arc in sedentary mice. In runners, a total of 21,573 BrdU- and 502 BrdU+ neurons were analyzed for the expression of Arc. In sedentary mice, the percentage of Arc expressing BrdU-neurons was approximately 0.5% (± 0.04 S.E.). No Arc expressing BrdU+ cells were identified in sedentary mice. The overall percentages of Arc expressing neurons was greater in the runners as compared to sedentary mice [Deviance=227.1, \( P<0.0001 \)]. In runners, the percentage of neurons expressing Arc was 1.9% (± 0.09 S.E.) in BrdU- neurons which was significantly different from the 3.8% (± 0.85 S.E.) in BrdU+ neurons [Deviance=7.3, \( P = 0.007 \)].

Localization of IEG-expressing new neurons within the granule layer

Collapsing data across all analyzed IEGs, a combined total of 2,197 BrdU+ and 125,885 BrdU- neurons were examined in two sub-regions of the granule cell layer of the dentate gyrus, the inside layer adjacent to the hilus versus the remaining layers, to determine the proportion expressing an IEG (either c-Fos, Arc, or Zif268). The BrdU+ neurons were concentrated in the inside layer adjacent to the hilus where 70% of the 1,433 neurons were located for runners and 81% of the 764 were located for sedentary animals. Thus, runners thus had a larger proportion of new neurons located away from the hilus than sedentary mice [Deviance=32.6, \( P<0.0001 \)]. In contrast, IEG-labeled cells were evenly distributed from the inside layer adjacent to the hilus to the outer edge of the granular layer (away from the hilus). In runners, the percentage of new neurons displaying an IEG was 2-fold greater if the new neurons were located in the middle of the granule cell layer as compared to adjacent to the hilus (Fig. 3.5) [Deviance=6.8, \( P=0.009 \)]. The same comparison could not be made in sedentary mice due to the low number of IEG expressing new neurons (2, 4, & 0 for c-Fos, Zif268, & Arc respectively).

Experiment 2: Neurogenesis and IEG induction in home cages without wheels

Home cage activity

Inspection of Figure 3.1B shows distance traveled in home cages increased slightly each day, after habituation to the home environment by day 2. The average distance traveled over the entire experiment was 0.44 km/day (± 0.019 S.E.).
Correlation between home cage activity and neurogenesis

Average daily distance traveled in home cages was not significantly correlated with the number of new neurons formed for each animal (see Fig. 3.6A). Pearson’s $r$ between average daily distance traveled and new neuron survival was 0.04 ($P>0.05$).

IEG induction from home cage activity

No significant correlations were observed between c-Fos, Zif268, or Arc expression and distance traveled in home cages during the respective IEG induction periods (see Fig. 3.6B, C, & D). Pearson’s $r$ for acute c-Fos, Zif268 and Arc induction and distance traveled was -0.13, 0.41, and 0.50 respectively (all $P>0.05$).

Experiment 3: IEG induction during the inactive light phase

c-Fos

Average distance traveled on running wheels 2 hrs before mice were euthanized was negligible and similar between groups sampled 3, 6, or 9 hrs after the onset of the light cycle. The collapsed average across all groups was 0.03 km (± 0.014 S.E.). c-Fos cell density did not differ between runners and sedentary mice. Average density for sedentary animals was 1.2 (± 0.36 S.E.) cells/mm³ (x 10³). Average density of c-Fos cells for runners sampled at 3, 6, 9 hrs after lights on were 1.0 (± 0.25 S.E.), 1.3 (± 0.15 S.E.), 0.8 (± 0.17 S.E.), respectively.

Zif268

Average distance run 4 hrs before mice were euthanized was negligible and similar between groups sampled 3, 6, or 9 hrs after the onset of the light cycle. The collapsed average across all groups was 0.2 km (± 0.14 S.E.). Zif268 cell density did not differ between runners and sedentary mice. Average density of Zif268 cells in sedentary mice was 6.4 (± 1.11 S.E.) cells/mm³ (x 10³). Average density for runners sampled at 3, 6, and 9 hrs after lights on was 8.0 (± 1.98 S.E.), 11.2 (± 1.10 S.E.), and 9.1 (± 0.55 S.E.), respectively.

Arc

Average distance run 8 hrs before mice were sampled either 3, 6, or 9 hrs after the onset of the light cycle was 2.2 km (± 0.62 S.E.), 0.6 km (± 0.07 S.E.), and 0.2 km (± 0.08 S.E.) respectively [F(2,15) = 8.54, $P = 0.003$]. Post hoc analysis revealed that mice
sampled 3 hours after onset of the light cycle ran significantly more than those sampled at 6 \([t(15)=1.9, P=0.012]\) and 9 hrs \([t(15)=2.2, P=0.006]\). However, mice sampled at 6 and 9 hours did not statistically differ from each other. Arc expressing cell density differed between groups \([F(3, 20) = 4.5, P=0.01]\). Post-hoc analysis revealed that runners sampled at 3 hrs had significantly more Arc cells than runners sampled at 6 hrs \([t(20)=3.1, P=0.03]\) and 9 hrs \([t(20)=3.3, P=0.02]\) after the onset of the light cycle. Sedentary mice were not significantly different from any running group. The average density of Arc cells for sedentary animals was 3.2 (± 0.36 S.E.) cells/mm\(^3\) (x 10\(^3\)). The average density of Arc cells for runners sampled at 3, 6, and 9 hrs after the onset of light cycle, was 5.1 (± 1.28 S.E.), 2.0 (± 0.28 S.E.), and 1.7 (± 0.52 S.E.), respectively.

**Discussion**

Results demonstrate that wheel running closely regulates the induction of three different IEGs in the hippocampal granule cell layer. The expression of c-Fos, Zif268, and Arc were all strongly correlated with the distance the animal traveled on wheels during the respective periods of induction for each IEG (see Fig. 3.2). The degree of IEG induction observed in the granule cell layer from wheel running is similar to previously reported inductions in mice performing spatial learning tasks or exploring novel environments (Stone et al., in press, Ramirez-Amaya et al., 2005, Kee et al., 2007, Trouche et al., 2009, Tashiro, 2007). Further, we report that an increased proportion of new dentate granule cells, 3-4 weeks old, were recruited into the neuronal c-Fos, Arc, and Zif268 induction from wheel running when compared to pre-existing or younger neurons (see Fig. 3.4). Arc, c-Fos, and Zif268 induction from running attenuated during periods of relative inactivity, suggesting these IEGs are induced from acute running and are not the result of long-term increases in basal concentrations within the granule cell layer. Taken together, these data suggest several IEGs are up-regulated in new and pre-existing neurons from acute bouts of physical activity.

While c-Fos, Zif268, Arc, and new neuron formation appear to be regulated by running (see Fig. 3.2), they are not strongly related to horizontal distance traveled in home cages without running wheels (see Fig. 3.6). The literature on the neurophysiology of home cage activity (without wheels) is scarce, however our lab recently reported no relationship when comparing distance traveled in home cages and number of new
neurons across 12 different inbred strains of mice. This is in contrast to the positive relationship ($r > 0.6$) observed when comparing distance traveled on running wheels and amounts of neurogenesis in the same 12 strains (Clark et al., 2011). Little is also known about whether IEGs can be induced in an activity-dependent manner in the granule cell layer by other forms of less robust voluntary movement. The current study demonstrates that in C57BL/6J females, a strain that commonly displays a strong positive relationship between running distance, IEG induction and neurogenesis in the hippocampus, do not display a relationship between distance traveled in home cages and neurogenesis or IEG induction. It is possible that a threshold level of physical activity is required before IEG induction and neurogenesis can be enhanced, such as that which taxes aerobic capacity (Pereira et al., 2007, Erickson et al., 2009, Chaddock et al. 2009).

The discovery that Arc is induced in neurons directly proportional to running intensity is particularly interesting because Arc expression in the hippocampus has largely been attributed to its role in learning and memory (Tzingounis and Nicoll, 2006, Bramham et al., 2008). The current study revealed that Arc is induced in the dentate gyrus of mice running on a fixed running wheel for over 30 days (see Fig. 3.2L). Thus, it is unlikely that learning was responsible for the Arc induction, as the animals were not experiencing any novel stimuli. To the best of our knowledge, this is the first example of Arc induction in the hippocampus from a voluntary behavior that appears to be absent of learning.

Evidence suggests that young neurons at a critical age, approximately between 1 and 6 weeks, become preferentially incorporated into behavior involving the hippocampus. Young adult-generated granule neurons display unique electrophysiological properties such as deceased threshold for LTP induction (Wang et al., 2000, Snyder et al., 2001, Schmidt-Hieber et al., 2004, Ge et al., 2007) suggesting new neurons may be more likely than older neurons to display an action potential during an event that activates the granule cell layer (Snyder et al., 2009a). Three recent reports using IEGs as neural activity makers following animal behavior might support this hypothesis, as they found IEGs were twice as likely to be induced in new neurons when compared to younger and pre-existing granule cells (Ramirez-Amaya et al., 2006, Kee et al., 2007, Clark et al., 2009). The current study extends those findings, as Arc, Zif68, and
c-Fos from wheel running were each more likely to become expressed in 3-4 week old new neurons, as compared to non-BrdU labeled NeuN cells which include mostly older pre-existing cells (see Fig. 3.4). On the other hand, a recent study used three different markers of cell division to directly compare older labeled neurons to younger labeled neurons and found a similar proportion of c-Fos induction from a hippocampal task in all neuron cohorts (Stone et al., in press). The authors concluded that a comparison of BrdU labeled neurons to unlabeled neurons, as was done in our study and previous studies, may be confounded as unlabeled neurons also contain a small percentage of immature neurons that have not yet become incorporated into hippocampal circuitry (Brandt et al., 2003, Kempermann et al., 2003, Clark et al., 2010). Hence, recruitment rates of mature neurons into IEG responses will be underestimated by sampling unlabeled cells because the sample also includes a small proportion of young non-functional NeuN cells. Given the recent Stone et al. findings, we cannot strongly conclude that new neurons are preferentially recruited into IEG responses to running. However, we can conclude that new neurons are recruited at a high rate into the IEG response.

New hippocampal neurons have been primarily studied for their potential role in learning. By reducing levels of neurogenesis or measuring the expression of IEGs in new neurons, studies have suggested that adult-born neurons are involved in several hippocampal-required learning tasks including spatial navigation (Snyder et al., 2005), contextual learning (Hernandez-Rabaza et al., 2009), novel object/place learning (Jessberger et al., 2009), pattern separation (Clelland et al., 2009), and trace conditioning (Shors et al., 2002). Moreover, other studies have suggested that new neurons from running play an important role in extending plasticity for spatial learning and memory (van Praag et al., 1999a, Clark et al., 2008). Under this context, wheel running appears to uniquely activate new neurons because it is seemingly unrelated to hippocampal-learning, since mice are habituated to running wheels and no new spatial information is being acquired.

It is possible that new neurons, characterized by their high degree of plasticity, become incorporated into whatever task the hippocampus is engaged in at any particular moment. Bland postulated an additional function of the hippocampus and related limbic structures is the processing of sensory or motor information (Bland, 1986, Oddie and
Bland, 1998). The second major class of cells in the rodent hippocampus is the theta cell, which fires action potentials with a frequency and amplitude proportional to the velocity and magnitude of voluntary movement (O'Keefe, 2007). For instance, theta-rhythm recorded in the hippocampus of wheel running rats displays a greater frequency and amplitude as the velocity of running increases (Oddie et al., 1996). Theta-rhythm induced from movement has been proposed to coordinate ensembles of cell activity involved in processing sensory cues necessary for maintenance of motor activity (Oddie and Bland, 1998, Bland and Oddie, 2001). Therefore, new neurons may be recruited into the function of the hippocampus during running by passively processing ongoing sensory information or actively modulating motor behavior (i.e. current intensity of activity). Recent studies using irradiation to reduce neurogenesis from running did not report any impairments in wheel running or motor task performance, so new neurons may not be critical for sensory or motor processing (Clark et al., 2008, Wojtowicz et al., 2008). However, neither study reported a complete ablation of neurogenesis. As technology improves for blocking neuron formation in the hippocampus, it would be interesting to test if a near complete removal of neurogenesis impairs running performance.

Running appears to increase the maturation and integration of new neurons into hippocampal circuitry. Precursor cells located in the inside layer adjacent to the hilus can differentiate into young neurons, and it has been suggested that as new neurons mature, they continue to move, or are pushed outwards to the superficial edges of the granule cell layer. Neurons located near the outer layers display a more pronounced dendritic arborization than neurons in the inner layer (Green and Juraska, 1985, Redila and Christie, 2006), suggesting that an increase in complexity of neuron morphology occurs with neuron age (van Praag et al., 2002, Schmidt-Hieber et al., 2004). In the current study, running mice had a greater proportion of new neurons located deep in granule cell layer than sedentary mice. These new neurons located in the middle of the granule cell layer were twice as likely to express either c-Fos, Zif268, or Arc from running when compared to the neurons located near the hilus (see Fig. 3.5). One explanation for these observations is that running may alter the maturation of a subset of new neurons located deeper in the granule cell layer, and that these neurons may be more likely to become functionally integrated. Additional support for this hypothesis has been demonstrated in
recent literature. Running increased the proportion of 21 day old neurons capable of expressing Arc following kainate-induced seizure, suggesting that a greater proportion of young neurons from running are integrated into hippocampal circuitry (Snyder et al., 2009b). Further, running increased the proportion of young neurons that expressed the protein NeuN, a commonly used marker for mature neurons (Clark et al. 2010). Our data in conjunction with previous reports suggest that a subset of neurons formed from running may develop more rapidly and incorporate into granule cell circuitry.

IEG expression from running-induced neural activity may be important for the increased survival and differentiation of new neurons from running. Several recent reports have revealed that neural precursor cells have activity sensing properties that are necessary for their differentiation into neurons (Deisseroth et al., 2004). We found wheel running increased the survival of granule cells in a manner strongly correlated with average distance the animal traveled each day (see Fig. 3.2C). Moreover, Arc, Zif268 and c-Fos were all induced from running in an activity-dependent manner (see Fig. 3.2F, I, & L). These relationships suggest that neural activity from wheel running may directly influence the survival of cells. Indeed, the expression of several genes that are transcribed by activity-induced IEG proteins also regulate hippocampal neurogenesis [e.g. neuropeptide Y by Zif268 (Wernersson et al., 1998), nerve growth factor by c-Fos (Herdegen and Leah, 1998, Kovacs, 1998), ect.]. Moreover, the expression of the brain derived neurotrophic factor (BDNF), which is necessary for the survival of newly formed neurons (Li et al., 2008), appears to regulate activity-dependant expression of the effector gene Arc (Bramham, 2008). It seems possible that IEGs are translating neural activity from wheel running into factors that promote the survival and differentiation of new neurons.

In conclusion, we report that acute bouts of wheel running induce the expression of three different IEGs, c-Fos, Zif268, and Arc, in the granule cell layer of the hippocampus. IEG induction and neurogenesis is specific for wheel running and does not occur for lower levels of physical activity such as that displayed in the home cage without running wheels. Moreover, new adult-born neurons are more likely to express c-Fos, Arc, and Zif268 from running than the population of pre-existing or younger neurons. These data, in conjunction with other reports, suggest that the granule cell layer
may play a role in passively processing ongoing sensory information or actively modulating motor behavior, and new neurons can be incorporated into this response. Whether new neurons generated from running are critical for processing sensory-motor information necessary for running, or whether they are also capable of being activated by hippocampus-dependant learning tasks are topics for future investigations.
Figure 3.1. Average wheel running and home cage activity. A) Average daily running distance in km/day (±SE) over 31 days of access to wheels for experiment 1. B) Average daily distance traveled in home cages without wheels in km/day (±SE) over 31 days as recorded by video tracking in experiment 2.
Figure 3.2. IEG induction and neurogenesis from wheel running. A) Representative coronal section stained for BrdU-DAB (combined with a light Nissl stain to highlight the dentate gyrus) of an animal euthanized in the sedentary condition (50X total magnification) B) same as A except representing the runner condition. C) Total number of new neurons shown for individual mice plotted against average daily running distance in km. D) Representative coronal section stained for c-Fos-DAB (combined with a light Nissl stain to highlight the dentate gyrus) of an animal euthanized in the sedentary condition (50X total magnification) E) same as D except representing the runner condition F) Number of c-Fos positive cells per cubic mm shown for individual mice plotted against distance run in km accumulated within 2 hrs before euthanasia. G)
Representative coronal section stained for Zif268-DAB (combined with a light Nissl stain to highlight the dentate gyrus) of an animal euthanized in the sedentary condition (50X total magnification) H) same as G except representing the runner condition I) Number of Zif268 positive cells per cubic mm shown for individual mice plotted against distance run in km accumulated within 4 hrs before euthanasia. J) Representative coronal section stained for Arc-DAB (combined with a light Nissl stain to highlight the dentate gyrus) of an animal euthanized in the sedentary condition (50X total magnification) K) same as J except representing the runner condition L) Number of Arc positive cells per cubic mm shown for individual mice plotted against distance run in km accumulated within 8 hrs before euthanasia. Sedentary mice are represented as grey circles and runners as black diamonds.
Figure 3.3. Distance traveled on wheels 12 hours before euthanasia for a representative low, medium, and high running mouse.
Figure 3.4. c-Fos, Zif268, and Arc expression in new neurons. A) Representative coronal section of the dentate gyrus stained for BrdU (green) and NeuN (blue), and c-Fos (red). The white boxes contain zoomed in images of two new neurons within the section.
that express c-Fos. B) The proportion of new and pre-existing granule neurons expressing c-Fos in both sedentary and runners. C) Representative coronal section of the dentate gyrus stained for BrdU (green) and NeuN (blue), and Zif268 (red). The white box contains a zoomed in image of a new neuron within the section that expresses Zif268. D) The proportion of new and pre-existing granule neurons expressing Zif268 in both sedentary and runners. E) Representative coronal section of the dentate gyrus stained for BrdU (green) and NeuN (blue), and Arc (red). The white box contains a zoomed in image of a new neuron within the section that expresses Arc. F) The proportion of new and pre-existing granule neurons expressing Arc in both sedentary and runners. ** $P < 0.001$ different from BrdU+ in runners. ^^ $P < 0.001$ different from either BrdU+ or BrdU- in runners.
Figure 3.5. Degree of IEG expression from running in new neurons depends on location within granule cell layer (GCL). The proportion of new neurons expressing an IEG located adjacent to the hilus compared to the middle of the GCL. Note that sedentary mice were not included in this comparison due to statistical limitations resulting from the small number of IEG expressing new neurons found (6 across all IEGs) despite an exhaustive search. ** indicates p<0.01
Figure 3.6. IEG induction and neurogenesis from home cage activity. A) Total number of new neurons shown for individual mice plotted against average daily distance traveled in home cages without wheels (km). B) Total number of c-Fos positive cells per cubic mm plotted against distance traveled in home cages without wheels (km) accumulated within 2 hrs before euthanasia. C) Total number of Zif268 positive cells per cubic mm plotted against distance traveled in home cages without wheels (km) accumulated within 4 hrs before euthanasia. D) Total number of Arc positive cells per cubic mm shown for individual mice plotted against distance traveled in home cages without wheels (km) accumulated within 8 hrs before euthanasia.
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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 BrdU to label dividing cells. The last 14 days mice were tested on water maze (2 trials per say for 5 days, then 1 hr later probe test), rotarod (4 trials per day for three days), and contextual fear conditioning (2 days), then measured for neurogenesis using immunohistochemical detection of BrdU and NeuN mature neuronal marker. Consistent with previous studies, in Sham animals, running increased neurogenesis 4 fold 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.
Introduction

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 to 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 have 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., 1995b), angiogenesis (Swain et al., 2003), gliogenesis (Li et al., 2005), and neurogenesis (van Praag et al., 1999b). 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., 1999a, Anderson et al., 2000, Van der Borgh 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, 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., 1999a, 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., 1999a, van Praag et al., 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 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 3 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., 1999a), 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., 2007a), 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 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., 1999a, van Praag et al., 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 Figure 4.1A.

**Husbandry**

Animals arrived at the Beckman Institute Animal facility from The Jackson Laboratory at 5 wks of age. Upon arrival they were housed 4 per cage by sex in standard polycarbonate shoebox cages with Bed-o-Cob™ bedding 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 (12:12 L:D; lights on at 7am and off at 7pm). 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.

**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 Figure 4.1B). Irradiated mice (n = 48) received 3 sessions of 5 Gy separated by 3- or 4-day intervals. In order to immobilize animals during irradiation, animals were anesthetized with an intraperitoneal 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, Rhodes et al., 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 x 20 x 14 cm (L W H) with a 23 cm diameter wheel mounted in the cage top. Dimensions of cages without wheels were 29 x 19 x 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 3 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 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, 2 trials per day for 5 days. A trial lasted either 60 sec or after the mouse reached the platform and remained on the platform for 10 sec. If a mouse did not reach the platform in 60 sec it was gently guided there by hand. Mice were placed back in their cage and allowed to rest for 30 sec between trials. One hour after training on day 5, the platform was removed and mice were tested with a probe trial (60 sec).

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 mL 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 vide tracking from above (black mouse on white background). Water temperature was maintained at 25-26 °C. Topscan (CleverSystems, Reston, VA) 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). 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 4 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 sec on day 1 and day 2. On day 1, mice in the fear group received 2 foot-shocks (0.5 mA duration 2 sec) at 120 and 150 sec. Mice in the control group did not receive any foot-shocks. On day 2, all mice were placed into the chamber for 180 sec without any foot-shocks. The chamber consisted of a plastic cage (dim 32 x 28 x 30 cm L W H) with a wire grid bottom connected to a shock scrambler controlled by digital timer (Med Associates, St. Albans, VT). 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 (ip) 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 micron 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 1-in-6 series of these sections (i.e., series of sections throughout the rostro-caudal extent of the brain with 240 micron increments separating each section, approximately 9 sections) were stained in each of the following ways.

1) 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% 20XSCC buffer, rinsed for 15 min in 10% 20XSCC buffer, then treated with 2N 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) at a dilution of 1:100 in TBS-X plus for 72 hrs 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) and stained using a diaminobenzidine kit (Sigma, St. Louis, MO).

2) 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), mouse anti-NeuN (1:50; Chemicon, Billerica, MA), 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 1:200 and also delivered as a cocktail, Cy2 anti-rat, Cy3 anti-mouse, Cy5 anti-rabbit. ABC and diaminobenzidine steps were omitted.

3) 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 hours at 4º C with primary antibody against cd68 (Serotec, Raleigh, NC) 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, Burlingame, CA) with diaminobenzidine as chromogen.

Image analysis
1) BrdU-DAB. The entire granule layer (bilateral), represented in the 1-in-6 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 10X (total 100X) 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 4 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.

2) Triple label. A total of 1,710 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).

3) 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 Figure 4.1C. 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 2 adjacent sections nearest the coordinates shown in Fig. 4.1C) under 10X 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 (sec) in the target quadrant of the water maze during the probe trial, duration (sec) 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 (4 levels, sham-sedentary, sham-runner, irradiated-sedentary, irradiated runner) as the between subjects factor: latency (sec), path length (m) and swim speed (m/sec) to the hidden platform, and latency to fall off the rotarod (sec) 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 Figure 4.1B. 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 x 4 cm square beam emitted from the source, exposed approximately 0.7 Gy/min through the 5 mm holes. This was true for only 3 out of 4 holes because 1 hole was accidentally not drilled at the correct angle of 1.65 degrees off the central axis during machining. Negligible radiation was detected around the edges of the shield.
All animals survived radiation (3 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 4.1 and Figure 4.1D. Exactly 111 days following radiation, when animals were euthanized (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 to sham.

**Wheel running**

See Figure 4.2. Radiation did not affect wheel running. Average distance traveled over the 54-day period was 5.7 km/day (± 0.36 SE) for irradiated mice and 5.8 km/day (± 0.42) for sham mice. Females ran 17% further than males (p<0.05).

**Neurogenesis**

See Figure 4.3. Running increased neurogenesis approximately 4 fold (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 to sedentary animals (interaction, p<0.05).

These changes in neurogenesis were attributed primarily to number of BrdU positive cells in the granular layer (Fig. 4.3A). 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. 4.3B). Radiation also slightly reduced proportion of new cells differentiated as glia (p<0.05).

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 strongly 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. 4.3C).
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 to 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 Figure 4.4.

Acquisition (Fig. 4.4A): 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., 1999a, 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 to 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 to 119.6 mm/s ± 3.2 (p<0.001) but did not differ between treatment groups.

Probe test (Fig. 4.4B): Each group, when analyzed separately, displayed significantly more time in the target quadrant as compared to 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 SE), and
similar length by day 5 (both 1.4 m ± 0.25 SE). 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 (13.7 cm/sec versus 12.5 cm/sec ± 0.34 SE; 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 sec and ending on day 5 with 11.8 sec, whereas males started at 45 and ended at 13.5 sec (± 1.7 SE). 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 Figure 4.5A.

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 Figure 4.5B.

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 to 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, Altman and Das, 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., 1999b), 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., 4 fold in this study, 4-5 fold in Rhodes et al. (2003a).
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. 4.2) but with reduced neurogenesis (Fig.
4.3). 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 intact neurogenesis suggests a specific contribution of new neurons to cognitive
\textit{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. 4.5A) or contextual fear (Fig. 4.5B). 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. 4.5B). 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. Mushi 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 (4 per cage) or in “enriched” cages (8 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., 1999a, van Praag et al., 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 meters per day to 4.7 km per day (Allen et al., 2001), whereas C57BL/6J run between 5 to 10 km/day (Fig. 4.2).

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. 4.4).

**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 central nervous system 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. 4.2) 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 4.1). This was expected based on previous work showing that inflammation subsides after approximately 2 months (Meshi et al., 2006). Results in Table 4.1 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. 4.4A) (van Praag et al., 1999a, Rhodes et al., 2003b, van Praag et al., 2005) 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. 4.4B) (van Praag et al., 1999a, van Praag et al., 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., 2006a, 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.
Table 4.1. 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

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Number microglia per mm³ ($\times 10^6$)</th>
<th>Size of microglia ($\mu$m³)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham Sodentary</td>
<td>Runner</td>
</tr>
<tr>
<td>Clingulate cortex</td>
<td>13.3 (1.54)</td>
<td>10.8 (1.00)</td>
</tr>
<tr>
<td>Lateral septum</td>
<td>15.0 (3.10)</td>
<td>8.9 (1.53)</td>
</tr>
<tr>
<td>Caudate</td>
<td>10.3 (2.62)</td>
<td>7.3 (1.24)</td>
</tr>
<tr>
<td>Dentate gyrus</td>
<td>14.7 (1.91)</td>
<td>12.3 (1.55)</td>
</tr>
</tbody>
</table>

$F$ values obtained from two-way ANOVA analysis.
Figures

Figure 4.1. 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 x 7.6 x 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 4 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 6-10 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.
Figure 4.2. 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.
Figure 4.3. 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.
Figure 4.4. 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 (sec) 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 (sec) 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.
Figure 4.5. Rotarod and contextual fear. A. Latency (sec) to fall off the accelerating rotarod over 3 days (each day the average of 4 trials), shown by group. B. Duration of freezing (sec) 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.
References


CHAPTER 5

New neurons generated from running are broadly recruited into different hippocampus-involved tasks.

(currently unpublished)
Abstract
Aerobic running increases the formation of new neurons in the adult rodent hippocampus. However, the function of new neurons generated from running is currently unknown. One hypothesis is that new neurons from running contribute to enhanced cognitive function by extending plasticity in the adult hippocampus. An alternative hypothesis is that new neurons from running preferentially or only contribute to processing information about wheel running behavior itself. The purpose of this experiment was to determine if new neurons generated from running become activated (as measured by Zif268 expression) in association with performance on other behavioral tasks that involve the hippocampus. The activation of 5-6 week old 5-Bromo-2’-deoxyuridine (BrdU) labeled new neurons were assessed in cohorts of running and sedentary adult C57BL/6J female mice following participation in one of three different tasks: the Morris water maze hidden platform task, novel environment exploration, or wheel running. Two hours after task performance all mice were euthanized by transcardial perfusion. Immunohistochemistry was performed on hippocampal sections with antibodies against BrdU, NeuN (mature neuron marker), and Zif268. Running doubled the survival of new neurons. The proportion of new neurons expressing Zif268 in running and sedentary mice was strongly related to the degree of Zif268 induction in the granule cell layer from each task. Running and sedentary mice displayed a nearly equivalent proportion of new neurons that expressed Zif268 following each task. Hence, runners had a greater overall recruitment of new neurons into each task than sedentary mice, demonstrating neurons formed during running can become “activated” by other hippocampal-involved behavioral tasks. Taken together, these results suggest that new 5-6 week neurons incorporated into hippocampal circuitry from running are not just preferentially “activated” by wheel running itself. Instead, new neurons from running are highly plastic units that become broadly recruited in different behavioral tasks.
Introduction

Regularly engaging in aerobic exercise can improve performance on tasks that involve the hippocampus in both humans and rodents (Chaddock et al., 2010, van Praag et al., 1999a, Rhodes et al., 2003b, Erickson et al., 2009, Greenwood et al., 2009). Although the mechanisms are not known, rodent models have suggested that cognitive enhancement could be due to the contribution of a variety of physiological changes occurring in the hippocampus from exercise including increased number of glial cells (Uda et al., 2006), more vasculature (Clark et al., 2009, Van der Borght et al., 2009), neurotrophic factors (Neeper et al., 1995), growth factors (Neeper et al., 1996), and changes in dendrite structure (Redila and Christie, 2006). One additional factor that may contribute to cognitive enhancement from exercise is the increased formation of new neurons in the granule cell layer of the hippocampus (van Praag et al., 1999b). This possibility is intriguing because the hippocampus is one of two brain regions that unarguably develops new neurons during adulthood (Gould, 2007).

While the function of new neurons in the adult hippocampus remains a mystery, it has been hypothesized that neurogenesis from running may contribute to enhanced cognitive function by extending plasticity in the adult hippocampus (van Praag, 2009). Studies have repeatedly demonstrated that animals with access to running wheels display improved performance on two tasks that involve the hippocampus including spatial learning (Rhodes et al., 2003b, Clark et al., 2009, van Praag, 2009) and contextual fear conditioning (Baruch et al., 2004, Clark et al., 2008, Greenwood et al., 2009). However, to date only one study has tested whether increased neurogenesis from running is necessary for these improvements (Clark et al., 2008). Our lab used gamma-irradiation focused at the hippocampus to reduce neurogenesis in running and sedentary mice. We found that running mice out-performed sedentary mice on contextual fear conditioning and rotarod, independent of irradiation treatment. However, only non-irradiated running mice displayed improved performance on the water maze over irradiated runners, irradiated sedentary, and non-irradiated sedentary mice. These data suggest new neurons from running-may improve spatial learning. However, irradiation induces side effects, some long lasting, that could potentially cause changes in cognitive performance (Nagai et al., 2000, Monje et al., 2003, Zhu et al., 2007). While these results provide promising
evidence that new neurons from running may contribute to improved cognitive function, it also emphasizes the necessity of additional models to test the function of new neurons from running.

An alternative hypothesis is that new neurons are specifically recruited into hippocampal circuitry related to the task that aided in their survival. Performance on several tasks that involve the hippocampus, including trace conditioning, Morris water maze, and enriched environment exploration, have been shown to enhance the survival of new neurons during a critical period of approximately 7-14 days in neuronal development (Gould et al., 1999, Ambrogini et al., 2000, Dobrossy et al., 2003, Leuner et al., 2004, Tashiro et al., 2007). Further, modest positive relationships are observed between the number of new neurons that survive and performance on hippocampal-dependent tasks (Ambrogini et al., 2000, Leuner et al., 2004, Dalla et al., 2007, Dalla et al., 2009), suggesting that a strong acquisition of a task may recruit more new neurons. Voluntary wheel running also causes a performance-dependent increase in survival of new neurons, as the number of neurons rescued is positively correlated with average distance traveled per day (Rhodes et al., 2003b, Bednarczyk et al., 2009, Clark et al., 2009, Clark et al., 2011a, Clark et al., 2011b). Thus, it is possible that new neurons from exercise in adult mice may preferentially function to support running activity itself.

Recent evidence from our laboratory suggests that new neurons generated from wheel running may function in processing information about the wheel running behavior. Using the expression of immediate early gene markers (IEG) of neuron activation, we have recently shown that wheel running massively increases neural activity in the granule cell layer strongly related to the distance the animal traveled before euthanasia (Clark et al., 2009, Clark et al. 2011a, Clark et al. 2011b). Moreover, new 5-6 week old granule neurons are more likely to become recruited into this response and display nearly a 2-fold increase in c-Fos induction when compared to pre-existing neurons (Clark et al., 2009). Whether or not new neurons generated from running can become activated during other tasks that engage the hippocampus is not known.

The goal of this study was to identify which hippocampal-involved tasks preferentially recruit new neurons formed from exercise. Immediate early gene (IEG) expression of Zif268 was used as the marker for neuronal activation in association with
task performance. The activation of 5-6 week old new neurons as well as older pre-existing neurons were assessed in cohorts of running and sedentary C57BL/6J mice following participation in three different hippocampus engaging tasks: the Morris water maze hidden platform task, novel environment exploration, and wheel running. Morris water maze represents a goal oriented spatial learning task. Novel environment exploration is a form of latent spatial learning in the absence of goal or reward. Wheel running represents a behavioral task that strongly induces IEG activation but is absent of learning.

Observing proportions and numbers of new neurons that display Zif268 in association with behavioral performance in running and sedentary conditions will provide insight into which new neurons (running induced or basally formed) are becoming recruited into the task. Mice housed in standard laboratory cages display a baseline amount of neurogenesis. Running extends basal neurogenesis by approximately doubling the survival of new neurons in C57BL/6J strain (Clark et al., 2011b). An equivalent or greater proportion of Zif268 expressing new neurons in running mice over sedentary mice during water maze or novel environment exploration would constitute evidence that new neurons from running function in the performance of other tasks. This is because runners will have more new neurons that display Zif268 than sedentary mice. Alternatively, if we observe a similar number of new neurons expressing Zif268 between sedentary and running mice (or a lesser proportion in runners than sedentary), this constitutes evidence that new neurons from running are not becoming activated during task performance. Under this alternative condition basally formed new neurons may more likely account for activation in both sedentary and runner groups. In combination with our previously published results showing new neurons from running are recruited into the IEG induction from running itself, this outcome would suggest that new neurons generated from running are preferentially recruited into processing information about wheel running behavior over other tasks.

We hypothesized that new neurons generated from running would display greater IEG activation than older neurons following wheel running, novel environment exploration, and Morris water maze hidden platform task. Our hypothesis is based on the idea is that new neurons are highly plastic units that become incorporated into whatever
the hippocampus is engaged in at any particular moment. However, it is possible that new neurons formed from running are only recruited into the running IEG response or are preferentially or only activated by wheel running. This hypothesis is based on the idea that new neurons are preferentially recruited in the specific tasks that aided in their survival.

**Materials and methods**

*Animals and husbandry*

Female C57BL/6J mice (n = 84 total) arrived at the Beckman Institute Animal Facility from The Jackson Laboratory at 5 weeks of age. Upon arrival, mice were housed 4 per cage in standard polycarbonate shoebox cages with corncob bedding (Harlan Teklad 7097 ¼ inch, Madison, Wisconsin, USA) for 4 weeks. Subsequently, mice were individually housed in either standard shoebox cages without running wheels, cages with running wheels mounted on top, or custom-made cages without running wheels for video tracking. Description and dimensions of cages are described in the materials section of each experiment. Rooms were controlled for temperature (21 ± 1°C) and photo-period (12:12 L:D; lights on at 9:00 AM and off at 9:00 PM). Food (Harlan Teklad, 7012) and water were provided ad libitum.

**Experiment 1: Persistence of new neuron survival following running**

The main objective of the entire study was to identify whether new neurons generated from running become recruited into behavioral performance of other tasks besides running. Because wheel running stimulates IEG expression (Rhodes et al., 2003a, Clark et al., 2009), animals cannot have access to running wheels in proximity to behavioral testing because IEG induction from wheel running would be difficult to distinguish from IEG induction from behavioral performance on the other tasks. However, if the newly added neurons persist after running has ceased for several days, we can study the recruitment of new neurons generated from running in behavioral performance on other tasks without the confounding influence of running itself. Therefore, the goal of this experiment was to determine if new neurons formed from running survive after running has stopped for a period of 12 days. We removed running wheels for 12 days instead of a few hours or days before behavioral testing to allow
animals to habituate to the new conditions and eliminate any possible effects of withdrawal from running on Zif268 induction

**Materials**

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

**Design**

Mice (9 weeks old) were divided into three groups. The first group (sedentary; n = 8) was individually housed in standard laboratory cages without running wheels for 42 days. The second group (continuous runners; n = 8) was individually housed in cages with running wheels for 42 days. The third group (runner; n = 8) was individually housed in cages with running wheels for 30 days and then moved to standard laboratory cages without running wheels for the remaining 12 days. The first 10 days, all mice received daily injections of 50 mg/kg bromodeoxyuridine (BrdU) to label dividing cells. On day 42, all mice were euthanized to quantify survival of new BrdU labeled neurons under each condition.

**Experiment 2: New neuron activation during behavioral task performance (see Figure 1)**

The purpose of this experiment was to identify whether new neurons generated from running become recruited into behavioral performance of other hippocampal engaging tasks besides wheel running (Clark et al., 2009, Clark et al., 2011a). Runners were individually housed in cages with running wheels for 30 days and then moved to standard laboratory cages without running wheels for the remaining 12 days (Fig. 1A). Sedentary mice were individually housed in cages without running wheels for the entire 42 days (Fig. 1A). The first 10 days, all mice received daily injections of BrdU (50mg/kg) to label dividing cells. During the final 12 days, separate cohorts of runner and sedentary mice performed three different behavioral tasks: novel environment exploration, Morris water maze hidden platform tasks, and wheel running (see Fig. 1B). On day 42, all mice were euthanized 2 hrs following final performance on each behavioral task. This period was chosen because Zif268 expression peaks approximately
2 hrs following a stimulus (Richardson et al., 1992, Zangenehpour and Chaudhuri, 2002). In order to estimate the correlation between physical activity and Zif268 expression in the granule cell layer, distance traveled while performing each task was recorded by video tracking.

**Behavioral tasks**

1) Novel environment exploration.

**Materials**

Custom made cages (without wheels) were constructed of clear plastic, to allow for overhead video tracking, with food and water access mounted on the side (see Clark et al., 2011a). Dimensions of the activity cages were 33.5 x 18 x16 cm (L W H). Video cameras mounted to the ceiling interfaced to a computer running TopScan software (CleverSys Inc., Vienna, VA) allowed for continuous monitoring of horizontal distance traveled in cages.

**Design**

Runners (n=8) and sedentary animals (n=8) were placed into the custom made cages for 2 hrs and then perfused. These groups will hereafter be referred to as novel runners, and novel sedentary animals. Since mice had not been previously exposed to activity cages or the surrounding visual cues, the environment was novel. Additional runner and sedentary mice were individually housed in the custom made cages continuously for the 12 days preceding euthanasia to serve as controls to detect Zif268 induced from the novel environment. These animals will hereafter be referred to as caged control runners and caged control sedentary animals. These mice were briefly removed and placed back into their respective cages by the experimenter 2 hours before euthanasia, to control for any potential Zif268 induction related to the handling of mice. Horizontal distance was continuously monitored using video tracking. All animals were euthanized, two hours after peak activity in the caged controls animals as determined by the video tracking data from the previous day. Peak daily activity occurred 6 hour post dark cycle onset.
2) Morris water maze.

Materials

Dimensions and parameters of the water maze followed (Clark et al., 2008). The maze consisted 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 mL 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 22 - 23 °C. Topscan (CleverSystems, Reston, VA) video tracking software was used to measure path length to platform or distance swam in the pool without platform.

Design

Runner (n=10) and sedentary mice (n=10) were trained on Morris water maze, for 3 days. These mice will hereafter be referred to as water maze runners and water maze sedentary animals. The first 2 days mice received 2 trials per day. On the third and final day, mice were given 3 trials, to assure maximal learning without physically fatiguing the mice from even more trials. Mice were trained on the water maze for 3 days, because previous experiments from our lab have demonstrated that mice reach asymptotic performance by day 4 and we wished to sample the animals at a time when they were still learning the maze (Clark et al., 2008). A trial lasted either 60 s or until the mouse reached the platform and remained on the platform for 10s. If a mouse did not reach the platform in 60s, it was gently guided there by hand. An additional group of runners (n=10) and sedentary animals (n=10) swam in the water maze without a platform and served as yoked controls for induction of IEGs from physical activity or non-goal oriented spatial learning. These groups will hereafter be referred to as yoked runners and yoked sedentary animals. Each mouse in these groups swam in the water for a duration equivalent to each trial of a randomly paired mouse navigating the water maze. Both water maze and yoked swim mice received an inter-trial interval of 30s for rest. Mice were placed into their home cages following the last trial. All mice were euthanized 2 hrs after the 1st trial of the last day.
3) Wheel running.

*Materials*
Same as experiment 1.

*Design*
Runners (n=6) and sedentary animals (n=6) mice were placed into cages with running wheels for 5 days (days 38 - 42) before euthanasia. These groups will hereafter be referred to as Run/Run and Sed/Run. Of note, in this experiment the runner condition was only removed from running wheels for 7 days before being placed back in cages with running wheels for the last 5 days. Mice were euthanized after 5 days of running, as opposed to a few hours or a single day of running to prevent Zif268 induction related to novelty of the wheel. All animals were euthanized 2 hrs after the peak of their daily running activity, as determined by an hourly average of the previous 4 days of running activity.

*Immunohistochemistry*
Following Clark et al. (2008), mice were anesthetized with 150 mg/kg sodium pentobarbital (ip) and then perfused transcardially with 4% paraformaldehyde in a phosphate buffer solution (PBS) 2 hours following novel cage exploration, water maze, or wheel running as described above. Brains were post-fixed overnight, and then transferred to 30% sucrose in PBS. Brains were sectioned using a cryostat into 40 micron coronal sections and stored in tissue cryoprotectant at -20°C. Separate 1-in-6 series of these sections (i.e., series of sections throughout the rostro-caudal extent of the brain with 240 micron increments separating each section) were stained in each of the following ways.
1) BrdU-DAB. Purpose: To detect BrdU-positive (newly divided) cells in the dentate gyrus. Free floating sections were washed in TRIS-buffered solution (TBS) and then treated with 0.6% hydrogen peroxide. To denature DNA, sections were treated with 50% de-ionized formamide, 10% 20XSCC buffer, 2N hydrochloric acid, and 0.1 M Boric acid. Sections were then treated with a solution of 0.3% Triton-X and 3% goat serum in TBS (TBS-X plus), and then incubated in primary antibody against BrdU made in rat (1:100; AbD Serotech, Oxford, UK) at a dilution of 1:100 in TBS-X plus for 72 hrs at 4 ºC. 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) and stained using a diaminobenzidine kit (Sigma, St. Louis, MO).

2) Zif268 -DAB. Purpose: To detect the presence of Zif268 in the granule cell layer. Free floating sections were washed in Phosphate-buffered solution (PBS) and then treated with 0.6% hydrogen peroxide. Sections were then treated with a solution of 0.2% Triton-X and 5% goat serum in PBS (PBS-X plus) for 1 hour, and then incubated in primary antibody against Zif268 (Santa Cruz Biotech, Santa Cruz, CA) made in rabbit at a dilution of 12:000 respectively in PBS-X plus for 48 hrs at 4 ºC. Sections were then washed in PBS, treated with PBS-X plus for 60 min and then incubated in secondary antibody against rabbit made in goat at 1:250 in PBS-X plus for 90 min at room temperature. Sections were then treated using the ABC system (Vector, Burlingame, CA) and stained using a diaminobenzidine kit (Sigma, St. Louis, MO).

3) Triple-fluorescent label. To determine the proportion of BrdU-positive (BrdU+) and BrdU-negative (BrdU-) neurons in the dentate gyrus that expressed Zif268. The procedure for BrdU-DAB was repeated except for the following. A cocktail was used for the primary antibody step that included rat anti-BrdU (1:100; AbD Serotech, Oxford, UK), mouse anti-NeuN (1:50; Millipore, Billerica, MA), and rabbit anti-Zif268 (1:2000; Santa Cruz Biotech, Santa Cruz, CA). Secondary antibodies made in goat were conjugated with fluorescent markers Cy2 anti-rabbit, Cy3 anti-rat, Cy5-anti mouse; Jackson ImmunoResearch, West Grove, PA) at dilution 1:200 and also delivered as a cocktail.

Image analysis

1) BrdU-DAB and Zif268-DAB. Following Clark et al., 2008, the entire granule layer (bilateral), represented in the 1-in-6 series was photographed by systematically advancing the field of view of the Zeiss brightfield light microscope, and taking multiple photographs, via camera interfaced to computer, under 10X (total 100X) magnification. Positively labeled cells in these photographs were counted by eye to generate unbiased estimates of total number of labeled cells (BrdU or Zif268). Positively labeled cells that were predicted to be in the top plane of the sections were not included in cell count estimates. The total counted of BrdU-labeled cells was multiplied by 6 to obtain
estimates of the total number of cells in the granule cell layer. In addition, the total volume of the dentate gyrus represented in the series was measured so that the Zif268 counts could be expressed per cubic micrometer dentate gyrus sampled. Area per section was obtained by outlining the entire bilateral granule cell layer. Volume was obtained by multiplying average area per section by the number of sections and then by the space between (240 microns) sections for each animal.

2) Triple label. Following Clark et al. (2009), a confocal Leica SP2 laser scanning confocal microscope (using a 40X oil HCX PL APO C5 objective with 1.25 numerical aperture, pinhole size 81.35 μm, 1-Airy Unit) was used to determine the proportion of BrdU cells (BrdU+) that differentiated into neurons (NeuN+) and to determine the proportion of neurons (BrdU+/NeuN+, or BrdU-/NeuN+) displaying Zif268. Each image sequentially captured a part of the dentate gyrus on a single plane (randomly selected on the z-axis) that was just out of view from previous image. This sequential imaging was done until the entire medial to lateral extent of the each bi-lateral granule cell layer was captured. The bi-lateral granule cell layer was imaged in 3 hippocampal sections (spanning the rostral, medial, and caudal extent) of 3 runner and 6 sedentary mice under each experimental condition, with the exception of the caged control condition where 3 runner and 3 sedentary mice were analyzed. Therefore, twice the amount of tissue was sampled for sedentary mice under each condition (except caged controls) to measure an approximately equivalent number of new neurons as runner mice. Only 3 sedentary and running mice were sampled in the cage control condition because the Zif268 induction was not task-specific and sparse, and thus would require an intense examination to obtain statistical power. All the new (BrdU+/NeuN+) and pre-existing (BrdU-/NeuN+) granular neurons, as well as the number of neurons co-labeled with Zif268 (BrdU-/NeuN+/Zif268+, or BrdU+/NeuN+/Zif268+) in that plane were counted for each image.

Zif268 expressing new neurons BrdU+/NeuN+/Zif268+ were also analyzed for the location within the granule cell layer. The number of IEG expressing new neurons that shared a border directly adjacent to the hilus of the granule cell layer was compared with the proportion of Zif268 expressing new neurons that had migrated into the granule cell layer (as defined by not having a border adjacent to the hilus). This analysis was
conducted to determine whether the probability of Zif268 expression in new neurons differs depending on the location of the new neurons within the granule cell layer.

Statistical Analysis

Data were analyzed using SAS, and R statistical software. In all analyses, P < 0.05 was considered statistically significant. The proportion of BrdU labeled cells in the granule cell layer that also expressed NeuN was compared in sedentary and runner conditions by logistic regression. To determine whether new neurons were more likely to display Zif268 from running, the proportions of BrdU-/NeuN+ and BrdU-/NeuN+ neurons expressing Zif268 were also compared by logistic regression. For these analyses, the deviance is reported in place of an F statistic.

The number of Zif268 cells per mm³ and total number of new neurons in the granule cell layer were compared between running and sedentary animals, as well as between each behavioral condition using ANOVAs. T-tests with Tukey correction were used for pair-wise group comparisons for statistically significant ANOVAs. An ANCOVA was conducted to determine whether the number of new neurons differed between runner and continuous runner mice in experiment 1 for a fixed level of running, i.e., after correcting for the correlation between distances traveled and number of new neurons. All correlations were estimated using simple linear or logarithmic regression.

Results

Experiment 1: Persistence of new neuron survival following running

Wheel running

The average distance traveled on wheels over the first 30 days for all mice was 5.5 (± 0.87 S.E.). Average daily running distance significantly differed between continuous runner and runner groups over the first 30 days of running [t(14) = 3.6, P=0.003]. Of note, 5 of the 8 highest running mice over the first 30 days were in the continuous runner group (see Fig 5.2B). Over the first 30 days, continuous runner mice averaged 7.5 km/day (± 1.05 S.E.), while runner mice averaged 3.5km/day (± 0.40 S.E.).

New Neuron Survival

Wheel running increased number of new neurons detected in the granule cell layer by approximately 2-fold relative to sedentary controls (Fig. 5.2A) [F(2,12) = 22.8, P < 0.0001]. Post hoc analysis revealed that continuous runners had slightly more new
neurons than runners \( t(21) = 3.1, P = 0.014 \). This subtle difference was due to significantly elevated running distance in continuous runners versus runners (see \textit{wheel running} section) during the first 30 days, and the observation that the average daily running distance was significantly correlated (Pearson’s \( r = 0.88 \)) with number of new neurons among individuals (Fig. 5.2B) \( [P < 0.001] \). Nonetheless, runners that were prevented from running the last 12 days displayed significantly more new neurons than sedentary mice \( t(21) = 3.6, P = 0.005 \). The percentage of BrdU cells double labeled with NeuN did not differ between continuous runner and runner mice and was 90\% (\( \pm 1.2 \)) in combined runners and 80\% (\( \pm 1.8 \)) in sedentary mice \( [\text{Deviance}=21.2, P<0.001] \).

Of note, because the ANCOVA revealed that neuron survival in runners did not differ from continuous runners for a fixed distance, only runners were compared to sedentary animals for the remaining experiments examining the activation of new neurons during hippocampal-involved tasks (see Fig. 1 for experimental design).

\textbf{Experiment 2: New neuron activation during behavioral task performance}

\textit{Novel environment exploration}

\textit{Locomotor activity}

Collapsing the data across runner and sedentary groups, mice exposed to the novel environment (novel group) displayed approximately a doubling of horizontal movement in activity cages 2 hrs prior to euthanasia over mice that habituated to the environment (caged controls group) (Fig. 5.3A) \( [t(13) = 5.9, P < 0.0001] \). Sedentary mice traveled slightly farther than runners in the novel environment (Fig. 5.3B) \( [t(13) = 2.2, P = 0.03] \). Caged control runner and sedentary mice traveled similar distances in the habituated environment.

\textit{Zif268 induction from novel environment exploration}

Mice exposed to the novel environment displayed a 2-fold increase in Zif268 over caged control mice (Fig. 5.3E) \( [F(1, 28) = 54.8, P < 0.0001] \). No significant main effect of exercise treatment or interaction between exercise treatment and novel environment exploration was observed. In novel environment exposed mice, Pearson’s \( r \) for the relationship between Zif268 density and total distance traveled over the 2 hour exposure
was 0.63 (Fig. 5.3F) \( (P = 0.009) \). In caged control mice, no relationship between Zif268 density and total distance traveled over 2 or 4 hours before euthanasia was observed.

**Morris water maze**

*Learning*

Both runners and sedentary animals learned the water maze as evidenced by decreased distance to reach the hidden platform over the three days of training (Fig. 5.4A) \( [F(2, 116) = 36.9, \ P < 0.0001] \). On the very first trial, more sedentary mice found the platform faster than runners by chance, thus sedentary mice maintained a slightly decreased distance to platform on average each day, as reflected in a significant main effect of exercise treatment \( [F(1, 116) = 10.2, \ P = 0.002] \). However, by day 3, sedentary and runner mice displayed an approximately equivalent distance to reach the platform. Average distance swam in the maze over the last 3 trials did not differ between the water maze and yoked swim groups.

**Zif268 induction in the granule cell layer from water maze**

Mice that performed the spatial water maze task displayed a modest, yet significant, increase in Zif268 expression over yoked swim mice (Fig. 5.4B) \( [F(1, 36) = 5.6, \ P = 0.02] \). There was no significant main effect of exercise treatment or interaction between exercise treatment and water maze group. No significant correlation was observed between Zif268 expression and average distance to reach the platform on the 3rd day of swimming for either the yoked swim or water maze group.

**Wheel running**

**Zif268 induction in the granule cell layer from running**

Running mice displayed a 4-fold increase of Zif268 over caged control mice (see Figs. 5.2C & 5.5A) \( [F(1, 28) = 94.7, \ P < 0.0001] \). Zif268 induction did not differ between sed/run and run/run groups (Fig. 5.5B). A significant correlation \( (r = 0.77) \) was observed between Zif268 density and total distance traveled (km) on wheels 2 hours before euthanasia \( (P = 0.008) \). However, Zif268 maintains a broad bell shaped curve and induction can likely be observed over a greater time period (Clark et al. 2011a). Pearson’s r was stronger at 0.94 for the relationship between of Zif268 density and total running distance 4 hours before euthanasia (Fig. 5.5C) \( (P < 0.0001) \).
The proportion of neurons (either BrdU+/NeuN- or BrdU-/NeuN+) expressing Zif268 was similar between sedentary and runner mice for each behavioral tasks (Fig. 5.6C). For both sedentary and runners, the percentage of neurons expressing Zif268 was approximately 2-fold greater in BrdU+ neurons than BrdU- neurons for each behavioral tasks (Fig. 5.6C & see Table 5.1 for post-hoc comparisons) [Deviance= 88.7, \( P < 0.0001 \)]. When combining runner and sedentary groups for each behavioral task, the proportion of new neurons that expressed Zif268 differed depending on location within the granule cell layer [Deviance= 27.3, \( P < 0.0001 \)]. Post-hoc analysis revealed that new neurons located deeper in the granule cell layer were more likely to display Zif268 than new neurons located directly adjacent to the hilus in yoked swim, water maze, and run conditions (see columns 9 & 10 in Table 5.1). For mice exposed to the novel environment, the proportion of new neurons located deeper in the granule cell layer that were labeled with Zif268 did not significantly differ from that of new neurons located directly adjacent to the hilus. However, it should be noted that both sedentary and running groups that were exposed to the novel environment displayed a trend towards a greater proportion of new neurons located deeper in the granule cell layer than adjacent to the hilus that displayed Zif268. Due to the overall low expression of Zif268 in caged control mice, there was not enough statistical power to analyze the proportions of BrdU+ cells expressing Zif268 based upon location in the granule cell layer.

To determine whether or not the proportional differences in Zif268 expression based upon location in the granule cell layer is unique to BrdU+ neurons, the same analysis was completed for all BrdU- neurons. The proportions of BrdU-/NeuN+ neurons that expressed Zif268 differed depending on location in the granule cell layer [Deviance= 300.0, \( P < 0.0001 \)]. Post-hoc analysis revealed that a larger proportion BrdU-/NeuN+ neurons located deeper in the granule cell layer also displayed Zif268 than BrdU-/NeuN+ neurons located adjacent to the hilus for both sedentary and runner mice in novel environment, yoked swim, water maze, and run conditions (see columns 7 & 8 in Table 5.1).

A strong and significant linear relationship (Pearson’s \( r = .88 \)) was observed between the degree of Zif268 expression in the granule cell layer (as reported in Figs.
5.3E, 5.4B, 5.5B) and the proportion of new neurons that displayed Zif268 for each task (Fig. 5.6D) \( (P < 0.0001) \). However, upon inspection of Figure 5.6D, the proportion of new neurons that display Zif268 appeared to be nearing asymptotic levels for tasks that more strongly activated granule cells (e.g. running). A stronger logarithmic relationship \( (r = .98) \) was observed between Zif268 expression in the granule cell layer and the proportion of new neurons that displayed Zif268 for each task (Fig. 5.6D) \( (P < 0.0001) \).

**Discussion**

The current study demonstrates that new neurons generated from running become “activated” during behavioral performance of other tasks that engage the hippocampus. The proportion of new neurons that expressed Zif268 was nearly the same in sedentary and running mice within each behavioral task (see Fig. 5.6C). Since runners had almost double the number of new neurons of sedentary mice (see Fig. 5.2A), approximately twice as many new neurons were recruited into task performance in running animals compared sedentary animals. This suggests that neurons generated from running are incorporated into the performance of other hippocampal-involved tasks. Moreover, the degree of Zif268 induction in the granule cell layer by each behavioral task was strongly correlated with the proportion of new neurons that expressed Zif268, independent of whether or not the mice were runners (see Fig. 5.6D). This suggests that a more active granule cell layer non-preferentially recruits a greater proportion of available new neurons. Taken together, these results favor the hypothesis that new 5-6 week neurons generated from running are highly plastic units that can be used during the performance of different hippocampus-involved tasks.

New neurons have a critical period in development, approximately at 7-14 days old, during which exposure to a hippocampal-involved task will increase the proportion of neurons that survive (Kitamura et al., 2010, Leuner et al., 2004, Tashiro et al., 2007). Interestingly, it has been shown that new neurons endure long after they are needed for the hippocampal-task that is associated with their survival (Leuner et al., 2004, Tashiro et al., 2007). In the current study, mice that were removed from access to running wheels after 30 days displayed only slightly fewer new neurons than mice that had continuous access to wheels the entire 42 days (see Fig. 5.2A). By chance, the continuously running mice traveled a greater distance on wheels over the first 30 days than mice that were
subsequently removed from wheels (see Fig. 5.2B). Since the number of new neurons formed is strongly correlated with daily running distance (see Fig. 2B) (Rhodes et al., 2003b, Bednarczyk et al., 2009, Clark et al., 2009, Clark et al., 2011a, Clark et al. 2011b), this likely represents a decreased formation of new neurons from running less, and not a loss of new neurons by mice that were removed from running wheels. These data suggest that new neurons generated during running persist and remain functional for at least 12 days after running has ceased. However, it is unknown if new neurons from running can survive for longer periods after running has stopped.

In the current experiment, new 5-6 week old neurons in both sedentary and runners were more likely to display Zif268 over primarily pre-existing neurons following water maze, novel environment exposure, and wheel running (see Fig. 5.6C). This result extends the finding of six other reports using immediate early genes (IEG) as neural activity makers following animal behavior, each finding new BrdU-labeled neurons were approximately 2 times more likely to display an IEG over populations primarily pre-existing neurons only labeled with NeuN (Stone et al., in press, Ramirez-Amaya et al., 2006, Kee et al., 2007, Tashiro et al., 2007, Clark et al., 2009, Clark et al. 2011b). It makes sense that new neurons would be more likely to display IEGs over pre-existing neurons for two reasons. First, studies have suggested that new neurons display unique electrophysiological properties that may result in an increased likelihood of firing an action potential over pre-existing neurons in response to an environmental stimulus (Wang et al., 2000, Snyder et al., 2001, Schmidt-Hieber et al., 2004, Ge et al., 2007). Secondly, while IEGs are widely used as markers for neural activity, they are commonly studied for the transcriptional regulation of proteins involved in cellular plasticity (Ramirez-Amaya, 2007). Thus, new neurons may be more likely to display Zif268 because they are more plastic during development while competing for incorporation into the granule cell circuitry with thousands of already integrated mature neurons.

On the other hand, a recent study used three different thymidine-analogue markers of cell division to directly compare a population of older labeled neurons to a distinct population of younger labeled neurons and found a similar proportion of c-Fos induction from a hippocampal task in each cohort of neurons (Stone et al., in press). The authors concluded that a comparison of BrdU labeled neurons to only NeuN labeled neurons, as
was done in our study and previous studies, may be confounded as NeuN expressing neurons also contain a small percentage of immature neurons that are presumably not yet incorporated into hippocampal circuitry (Brandt et al., 2003, Kempermann et al., 2003, Clark et al., 2010). Hence, recruitment rates of mature neurons into IEG responses may be under estimated by sampling only NeuN cells because the sample also includes a small proportion of young, presumably non-functional cells. In the current study, under each experimental condition there was proportionally more Zif268 expression in BrdU-/NeuN+ cells located away from the hilus as compared to directly adjacent to the hilus (see Table 5.1), which may suggest that more BrdU-/NeuN+ neurons closer to the hilus are younger and not yet functionally integrated. Given our results and the recent Stone et al. (in press) findings, it is difficult to strongly conclude that new neurons are preferentially “activated” or display Zif268 during hippocampal-task performance. However, we can conclude that new neurons in sedentary and running mice are recruited at a high rate into the Zif268 response following novel environment exploration, water maze, and wheel running.

Live cell recordings have demonstrated that new 1-6 week neurons display a decreased threshold of LTP induction (Wang et al., 2000, Snyder et al., 2001, Schmidt-Hieber et al., 2004, Ge et al., 2007). Taken together with our findings that runners have a greater incorporation of new neurons into the performance of different behavioral tasks, these data suggest that new neurons from running may contribute to the enhanced performance on hippocampal-involved tasks. Indeed, studies have repeatedly shown that exercising rodents display improved performance on tasks that engage the hippocampus (Creer et al. 2010, van Praag et al., 1999a, Rhodes et al., 2003b, Clark et al., 2008, Clark et al., 2009, Greenwood et al., 2009). Further, our lab has recently shown that enhanced water maze performance is compromised in running mice when hippocampal-neurogenesis is reduced, providing further support for the hypothesis that the increased formation of new neurons contributes to better spatial-learning (Clark et al., 2008). In the current study, we failed to replicate the improvement in performance on the water maze from running, as it would appear at first glance that running mice did not outperform sedentary mice. However, on the first trial a much larger number of sedentary mice were able to locate the platform on their own by chance, leading to a substantially disparate
starting point for running and sedentary mice. This makes it impossible for the current study to assess whether mice benefited from running on water maze performance. Nevertheless, both runner and sedentary groups displayed excellent learning, which is sufficient to address the hypothesis that new neurons are activated during water maze performance.

Increased IEG expression in hippocampal granule cells of animals performing the spatial water maze over controls animals (either swimming in a maze without a platform or with a visible platform) could be the result of hippocampal-dependent spatial learning. However, literature has been mixed as to whether an increase in IEG expression above water maze controls is observed (Guzowski et al., 2001, Snyder et al., 2009a, Snyder et al., 2009b, Trouche et al., 2009). Depending on water maze paradigm, varying results could be related to confounding factors that also influence granule cell IEG expression, such as stress or physical activity (Titze-de-Almeida et al., 1994, Clark et al. 2011a). It is also important to keep in mind that mice are receiving spatial information about the water maze environment whether or not they need to use the spatial cues to locate a hidden platform. Therefore, the hippocampus is likely engaged in water maze controls, much like mice briefly exploring a novel environment (McNaughton et al., 1983). In the current study, we limited the number of exposures to the maze to three brief trials before euthanasia in order to decrease stress and physical activity from several consecutive trials on one day in the maze. A small but significant increase in Zif268 was observed in mice performing the water maze task, as compared to yoked swim controls (see Fig. 5.4B). It is possible that this subtle increase in Zif268 could reflect the active use of spatial information to locate the platform in the water maze group, as opposed to the passive acquiring of spatial information by yoked swim controls. However, because we did not observe a relationship between distance to reach the platform and Zif268 expression, it is difficult to strongly conclude what is creating the increase in Zif268 from water maze performance above swim controls.

Whether or not the increase in Zif268 expression while exploring the novel environment is due to formation of spatial representations, physical activity, general arousal, stress-related or a combination of these factors is difficult to determine. Zif268 from physical activity and formation of spatial representations in a novel environment are
difficult to distinguish because animals that are more active in a novel environment will likely be taking in more spatial information. In the current study, Zif268 expression in caged control mice (not exposed to a novel environment) was not related to distance traveled two or four hours before euthanasia. However, in novel mice, a positive relationship was observed between the degree of Zif268 expression and distance traveled over the 2 hour exposure to the novel cage (see Fig.5.3F). These data may suggest that Zif268 is becoming induced by the formation spatial representations and not from general locomotor activity, as mice that traveled farther in a novel chamber are acquiring more spatial information than caged control mice that were already familiar with the environment. On the other hand, our lab has recently found that IEG expression is related to distance traveled on running wheels and not distance traveled in standard laboratory cages in proximity to sacrifice (Clark et al. 2011a). Since mice travel much farther on wheels than in standard laboratory cages without wheels, this may suggest that a threshold of movement must be met before IEGs become induced in a physical activity-dependent manner in the granule cell layer. In the current study, novel environment exposed mice traveled nearly twice as far as caged controls the 2 hrs before euthanasia, and thus may have exceeded a threshold of movement necessary to induce Zif268 in granule cells. Therefore, it is difficult for the current study to conclude whether Zif268 expression while exploring a novel environment is a result of physical activity, the formation of spatial representations, or a combination of several factors. Future work is needed to tease apart the factors contributing to IEG expression in the granule cell layer while exploring a novel environment.

In conclusion, the current data provide evidence that new 5-6 week neurons generated from running are highly plastic units that can respond to distinct tasks that engage the hippocampus. Further, running mice have more Zif268 expressing “activated” new neurons than sedentary mice. One of the most basic ways to address whether new neurons from running can extend plasticity in the hippocampus that contributes to performance on other tasks is to provide evidence that new neurons are becoming activated during performance on a particular task. However, one limitation of the use of IEGs is that it is difficult clearly determine what information these “activated” neurons are processing. There are several hypothesized functions of new neurons
including sensory or motor processing (Clark et al., 2009, Clark et al. 2011a), temporary units of memory storage (Kitamura et al., 2009), pattern separation (Clelland et al., 2009), mood regulation (Sahay and Hen, 2008), etc. It is possible that new neurons become activated during a behavioral task to perform one or all of the previously listed functions. As methods of blocking neurogenesis continue to improve, we can continue to discover the potential functions of new adult born hippocampal neurons.
Table 5.1. The total number of cells counted, percentage NeuN+/BrdU- & NeuN+/BrdU+ cells co-labeled with Zif268 (S.E.), percentage of NeuN+/BrdU- cells co-labeled with Zif268 (S.E.) based upon location in the granule cell layer, and the percentage of NeuN+/BrdU+ cells co-labeled with Zif268 (S.E.) based upon location in the granule cell layer for each behavioral task and exercise treatment.

<table>
<thead>
<tr>
<th>Task</th>
<th>Exercise</th>
<th># Cells Counted</th>
<th>% Cells w/ Zif268</th>
<th>% BrdU-/NeuN+ w/ Zif268</th>
<th>% BrdU+/NeuN+ w/ Zif268</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>in GCL*</td>
<td>by hilus</td>
</tr>
<tr>
<td>caged control</td>
<td>Sedentary</td>
<td>16,199</td>
<td>0.6 (± 0.05)</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>caged control</td>
<td>Runner</td>
<td>20,103</td>
<td>0.6 (± 0.05)</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>novel</td>
<td>Sedentary</td>
<td>42,590</td>
<td>1.7 (± 0.09)</td>
<td>1.6 (± 0.07)</td>
<td>6.4 (± 1.75)</td>
</tr>
<tr>
<td>novel</td>
<td>Runner</td>
<td>23,626</td>
<td>1.4 (± 0.06)</td>
<td>1.9 (± 0.09)</td>
<td>4.5 (± 1.78)</td>
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<tr>
<td>yoked swim</td>
<td>Sedentary</td>
<td>37,179</td>
<td>1.7 (± 0.09)</td>
<td>2.2 (± 0.09)</td>
<td>6.5 (± 1.72)</td>
</tr>
<tr>
<td>yoked swim</td>
<td>Runner</td>
<td>13,084</td>
<td>2.0 (± 0.07)</td>
<td>2.3 (± 0.11)</td>
<td>6.5 (± 1.80)</td>
</tr>
<tr>
<td>MWM</td>
<td>Sedentary</td>
<td>33,660</td>
<td>2.1 (± 0.11)</td>
<td>2.3 (± 0.11)</td>
<td>6.5 (± 1.80)</td>
</tr>
<tr>
<td>MWM</td>
<td>Runner</td>
<td>19,718</td>
<td>2.3 (± 0.08)</td>
<td>2.5 (± 0.09)</td>
<td>6.5 (± 1.64)</td>
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<tr>
<td>Run</td>
<td>Sedentary</td>
<td>36,925</td>
<td>2.4 (± 0.11)</td>
<td>2.7 (± 0.10)</td>
<td>6.4 (± 1.46)</td>
</tr>
<tr>
<td>Run</td>
<td>Runner</td>
<td>19,719</td>
<td>2.4 (± 0.11)</td>
<td>2.8 (± 0.10)</td>
<td>11.2 (± 1.55)</td>
</tr>
</tbody>
</table>

*a P < 0.01 from % Zif268 in NeuN+/BrdU-

*b P < 0.0001 from % Zif268 in NeuN+/BrdU- in GCL

*c P < 0.05 from % Zif268 in NeuN+/BrdU+ in GCL for combined runner & sedentary exercise groups

*d P < 0.001 from % Zif268 in NeuN+/BrdU+ in GCL for combined runner & sedentary exercise groups

*GCL is an abbreviation for granule cell layer

na was not quantified due to low Zif268 expression
Figure 5.1. Experimental design. A) The running and sedentary treatment groups used in experiments 2, 3, & 4. All mice received daily BrdU injections the first 10 days and were euthanized two hours after behavioral testing on day 42. B) A depiction of the behavioral testing design which includes behavioral treatments, sample sizes for a runner or sedentary group, brief description of treatments, and the days each task was performed.
Figure 5.2. New neuron formation from running in experiment 1. A) Average total number of BrdU cells co-labeled with NeuN (±SE) in the granular layer of the dentate gyrus in mice that were housed in standard laboratory cages for 42 days (Sedentary), cages with running wheels for 30 days and then moved to standard laboratory cages for 12 days (Runner), and cages with running wheels for 42 days (Continuous Runner). B) The relationship between average distance traveled on running wheels during the first 30 days and the number of new neurons formed. ** $P < 0.01$ from sedentary, ^$P < 0.05$ from runner.
Figure 5.3. Induction of Zif268 from novel environment exploration. A) Average distance (±SE) traveled in the activity cages 2 hours before euthanasia for mice that were continuously housed in the environment for 12 days (Caged Control) and mice that were exposed to the environment for the first time (Novel). B) Average distance (±SE) traveled in activity cages 2 hours before euthanasia for novel sedentary and runner mice. C) Representative coronal section from a caged control mouse stained for Zif268-DAB (combined with a light Nissl stain to highlight the dentate gyrus.) D) same as C) except for a mouse from the novel groupE) Average number of Zif268 labeled cells (±SE) per cubic mm in the granular layer of the dentate gyrus. F) Relationship between distances traveled 2 hours before euthanasia and number of Zif268 labeled cells per cubic mm in the granular layer. * $P < 0.05$, **$P < 0.01$ from control
Figure 5.4. Induction of Zif268 from Morris water maze.  A) Average distance traveled to the hidden platform (+/- SE).  B) Average number of Zif268 labeled cells (±SE) per cubic mm in the granular layer of the dentate gyrus of sedentary and runner mice that performed the spatial water maze task (WM) or swam in the pool without a platform with the latency matched to an individual from the water maze group (Yoked).  C) Representative coronal section from a yoked swim mouse stained for Zif268-DAB (combined with a light Nissl stain to highlight the dentate gyrus.)  D) same as C) except for a mouse from the water maze group. * $P < 0.05$
Figure 5.5. Induction of Zif268 from running. A) Representative coronal section from a runner stained for Zif268-DAB (combined with a light Nissl stain to highlight the dentate gyrus). B) Average number of Zif268 labeled cells (±SE) per cubic mm in the granular layer of the dentate gyrus of sedentary and runner mice that were placed into cages with free access to running wheels for the final 5 days. C) Relationship between the distances traveled on running wheels 4 hours before euthanasia and the numbers of Zif268 labeled cells per cubic mm counted in the granule layer of the DG.
Figure 5.6. Induction of Zif268 expression in new versus pre-existing neurons. A) Representative coronal section of the dentate gyrus stained for BrdU (green), NeuN (blue), and c-Fos (red) of a mouse that was on running wheels 2 hrs before euthanasia. B) A zoomed in image of the white box in A) with arrows pointing to two new neurons expressing Zif268 that were located adjacent to the hilus. C) Percentage of BrdU-/NeuN+ and BrdU+/NeuN+ neurons that displayed Zif268 (±SE) for both runner and sedentary mice following performance on each behavioral task. D) Density of Zif268 expression in the granule cell layer plotted against the percentage of new neurons that displayed Zif268 for each behavioral testing group.
References


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