Research report

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

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A B S T R A C T

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 h 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 h 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.

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1. 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 [5,8], humans [1,11], and non-human primates [15]. 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., [12,16,24]). One potent environmental factor that increases neurogenesis is aerobic exercise [38]. 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 [4,6,7,31,38]. 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 [6,31].

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 [13,21,32]. 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 [13,19,22,26,37]. In C57BL/6J male mice, we reliably find levels of running reach a peak at approximately day 20 and thereafter maintain a plateau [6,7]. 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. [21] examined proliferation and survival of new neurons at three different time-points

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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 [3,6,27,30]. Recently, we discovered that new 7-week old neurons are more likely than older neurons to display c-Fos from running [6]. These data suggest that activation of granule neurons from running is probably related to the signaling for increased neurogenesis. Although it is known that c-Fos induction from running persists after as many as 40 days of continuous access to exercise wheels [6], whether c-Fos expression is greater when running has reached a plateau versus during the initial escalation period is not known.

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 four 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 [6,27,30], 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 [23], the exercise-induced enhancement of neurogenesis and activation of the dentate gyrus may continue at a stable rate, or possibly decrease over time.

## 2. Materials and methods

2.1. 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 four per cage in standard polycarbonate shoebox cages with corncob bedding (Harlan Teklad 7097 1/4 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 $36\,cm \times 20\,cm \times 14\,cm$ ($L \times W \times H$) with a 23 cm diameter wheel mounted in the cage top (Respironics, Bend, OR). Cages without wheels were $29\,cm \times 19\,cm \times 13\,cm$ ($L \times W \times H$). 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 ($21 \pm 1\,°C$) 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.

2.2. Experimental design

Mice were randomly assigned to six groups ($n = 6$ per group) as shown in Fig. 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 h after the last BrdU injection to measure cell proliferation and early neuronal differen-
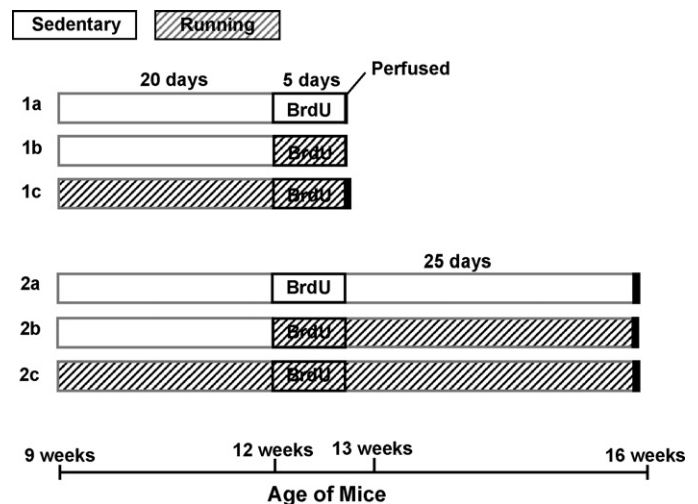
Fig. 1. Experimental design. Group 1 was used to measure proliferation and group 2 survival of new neurons born in non-running controls (sub-group a), at the onset of access to running wheels (sub-groups b), and 20 days after the onset of access to wheels during peak running distance (sub-groups c). 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.

tiation (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 h after the onset of the dark phase of the light/dark cycle, which is approximately 1.5 h 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 min after cell activation.

2.3. 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 μm 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 μm increments separating each section) were stained in each of the following ways.

2.3.1. BrdU-DAB and Ki67-DAB
2.3.1.1. Purpose: cell proliferation and mitotic markers. Free floating sections were washed in tris-buffering 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% 20× SCC buffer, 2 N 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 h at 4 °C. Sections were then washed in TBS, treated with TBS-X plus for 30 min and then incubated in secondary antibody against rat made in goat at 1:250 in TBS-X plus for 100 min at room temperature. Sections were then treated using the ABC system (Vector, Burlingame, CA) and stained using a diaminobenzidine kit (Sigma, St. Louis, MO).

2.3.2. Triple-fluorescent label
2.3.2.1. Purpose: to determine the proportion of BrdU-positive cells (BrdU+) in the dentate gyrus that differentiated into neurons. The same procedure as in Section 2.3.1 was used except a cocktail was used for the primary antibody step, rat anti-BrdU (1:100; 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.
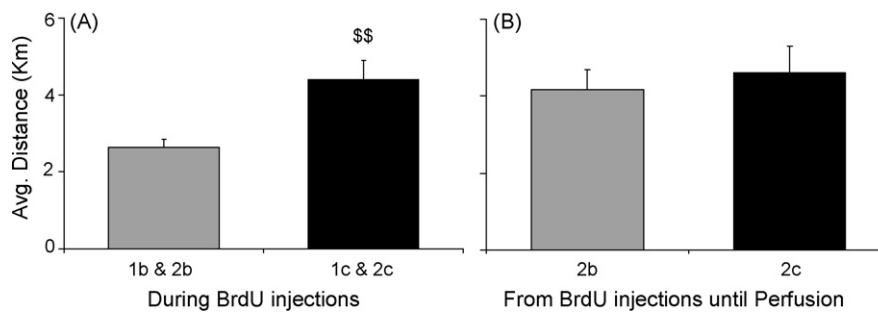
Fig. 2. Average wheel running distance between groups. (A) Average daily running distance in km/day (±SE) over 5 days of BrdU injections shown separately for the group given BrdU injections at the onset of access to running wheels versus after 20 days of running. (B) Average daily running distance in km/day (±SE) over 30 days starting at the onset of BrdU injections until euthanasia. The x-axis represents groups as shown in Fig. 1. $$ indicates P < 0.01.

2.3.3. c-Fos-DAB

2.3.3.1. 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 h, 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 h 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 1:500 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).

2.4. Image analysis

2.4.1. BrdU-DAB, Ki67-DAB and c-Fos-DAB

Following [7], 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 10× (total 100×) 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 μm^3 dentate gyrus sampled.

2.4.2. Triple label

A confocal Leica SP2 laser scanning confocal microscope (using a 40× 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 μm^3 per mouse was calculated as the number of BrdU cells per μm^3 multiplied by average proportion BrdU cell co-expressing NeuN for the designated group (groups as listed in Fig. 1).

2.5. 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 mm³ of dentate gyrus were compared between groups (shown in Fig. 1) using one-way ANOVA. T-tests were used for post hoc analysis.

The number of c-Fos cells per mm³ 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 was 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/h) and number of cells expressing c-Fos were estimated using simple linear regression.

3. Results

3.1. 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 (Fig. 2 in [7] and

Fig. 1 in [6]). 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. 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. 2B). The average level of wheel running over the entire experiment for all mice was 4.56 km/day (±0.10 SE).

3.2. Neurogenesis
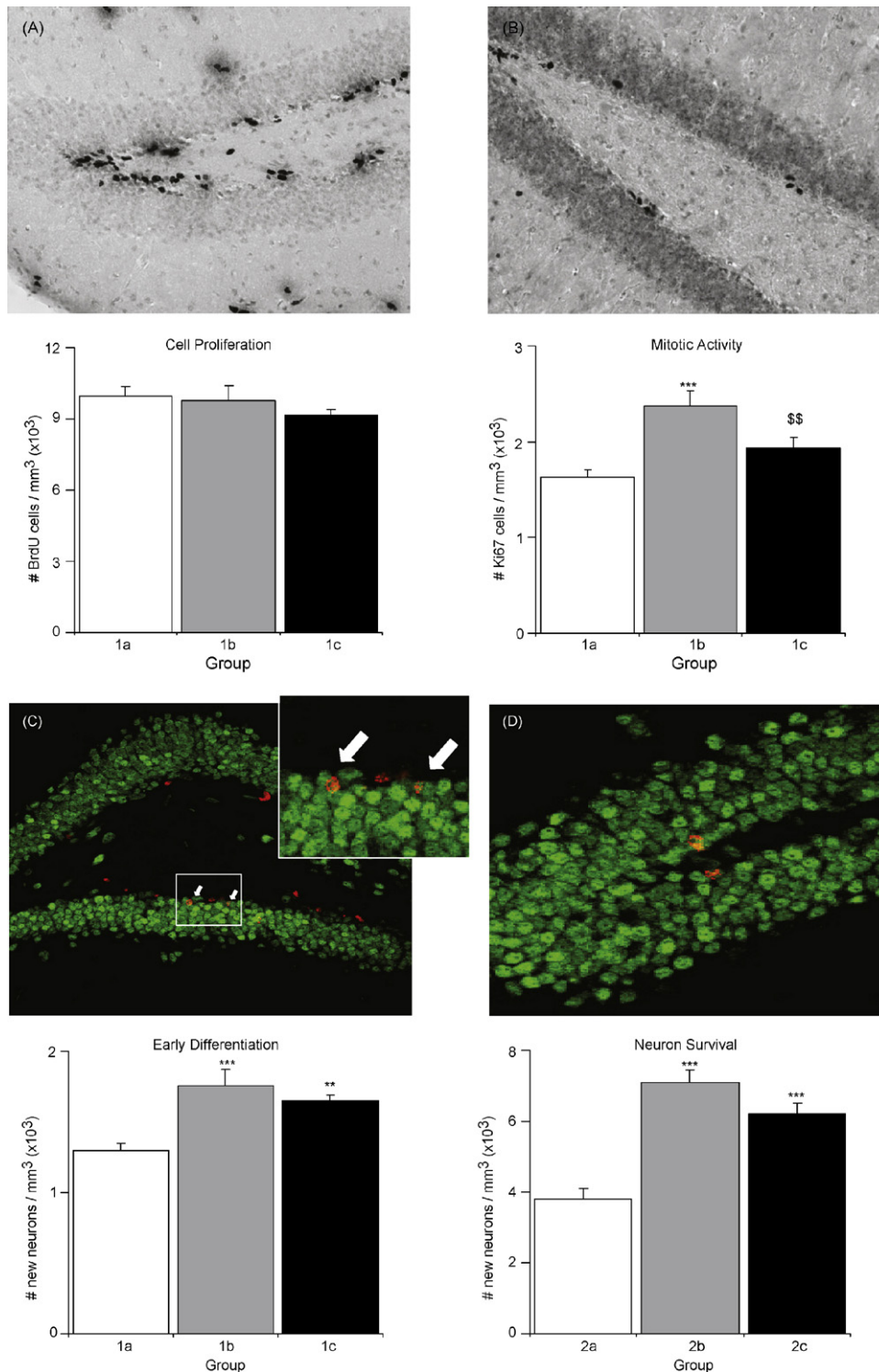
3.2.1. Cell proliferation

The number of proliferating BrdU-labeled cells measured 24 h after the last injection did not differ across groups (Fig. 3A). However, running increased the number of cells labeled with Ki67 in the sub-granular zone of the dentate gyrus (Fig. 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].

#### 3.2.2. Early determination

Running increased the proportion of BrdU cells double labeled with the neuronal marker NeuN, 24 h after the 5th day of BrdU injections [deviance(1,16) = 5.37, P = 0.02]. The percentage of BrdU cells co-labeled with NeuN, 24 h 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. 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.

3.2.3. 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. 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 [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.

Fig. 3. Proliferation, mitosis, early differentiation and survival of new neurons. (A) Top: Representative coronal section of the dentate gyrus stained for BrdU-DAB, 24 h after the last BrdU injection. Bottom: Average number of BrdU-labeled cells ($\pm$SE) per mm^3 in the granular layer of the dentate gyrus, measured 24 h 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 ($\pm$SE) per mm^3 in the sub-granular zone. (C) Top: Representative coronal section of the dentate gyrus stained for BrdU (red) and NeuN (green), 24 h after the last BrdU injection. The arrows indicate double labeled cells. Bottom: Average number of BrdU-labeled cells co-expressing NeuN ($\pm$SE) per mm^3 in the granular layer of the dentate gyrus, 24 h 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 ($\pm$SE) per mm^3 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 Fig. 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. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
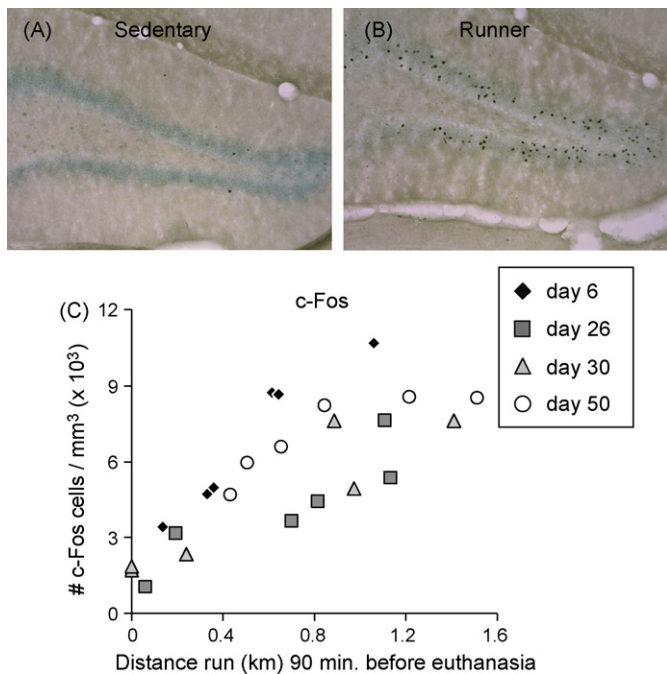
Fig. 4. c-Fos induction from wheel running. (A) Representative coronal section stained for c-Fos-DAB (combined with a light Nissl stain to highlight the dentate gyrus) of an animal euthanized in the sedentary condition (50× total magnification). (B) Same as A for an animal running before euthanasia. (C) Number of c-Fos positive cells per mm³ 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 min before euthanasia. Day 6 had a significantly steeper slope than all other days sampled.

3.3. 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. 4A and 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. 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 $P \leq 0.02$). 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. 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. 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.

4. Discussion

The main finding of this study is that proliferation (Fig. 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. 3C) and survival (Fig. 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 [13,21,32].

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 [6]. 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. 2A), the survival of new neurons labeled later, during higher levels of running displayed a slight, statistically nonsignificant, reduction as compared to earlier when levels of running were lower (Fig. 3C and 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. 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 [13,21,32]. 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 [38].

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 [13,21,32]. Notably, we found no increase in the number of BrdU cells when assessed 24 h after the last injection at the onset of access to running wheels or during peak running (Fig. 3A), even though we observed a significant increase in differentiation toward the neuronal lineage (Fig. 3C), and an approximate doubling in survival of new neurons after 25 days (Fig. 3D).

One plausible explanation for the discrepancy between BrdU (Fig. 3A) and Ki67 (Fig. 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 [18]. 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 and 17 days in C57BL/6 mice [18,21,37]. 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 [18,37,39]. 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 h. This could return the total number of early surviving proliferating cells (as measured by BrdU 24 h after the last injection) in running mice to levels similar to that of sedentary animals (Fig. 3A).

Results of this study (Fig. 3C) confirm previous reports showing that neuronal differentiation begins within the first 5 days of birth of new neurons [17]. In this study, 12–18% of BrdU cells 24 h 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

[7,38]. 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 [9,20,33,36]). 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 [6], and in the present study 50 days (Fig. 4C). In response to mild forced exercise on a treadmill consisting of walking at a speed of 0.48 km/h 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 [23]. 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 [23]. 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. 3B) and a slight increase in cell survival (Fig. 3C and D). With continued running, decreased proliferation decreased survival of new neurons paralleled the slightly attenuated c-Fos response to wheel running (Fig. 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 [10]. Excitation of progenitor cells, in vitro, increases differentiation into the neuronal lineage [10]. The location of neural progenitor cells in the sub-granular 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 [6].

Recent work has implicated the neurotransmitter *gamma*-aminobutyric acid (GABA) in the activity-dependent differentiation of progenitor cells into new neurons [25]. GABA interneurons are the first cell type to establish connections with new born granule cells [14,28]. Due to intracellular differences in ion concentrations between mature neurons and progenitor cells, GABA has an excitatory effect on progenitor cells [29]. 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 [35]). Many hippocampal glutamatergic mossy fibers and theta rhythm regulating medial septum projections also innervate GABAergic interneurons in the dentate gyrus [2,34]. 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 [13,21,32], 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.

Acknowledgements

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