Brain, Behavior, and Immunity 26 (2012) 803–810

Contents lists available at SciVerse ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Wheel running attenuates microglia proliferation and increases expression of a proneurogenic phenotype in the hippocampus of aged mice

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ARTICLE INFO

Article history:
Available online 25 October 2011

Keywords:
Exercise
Proliferation
Hippocampus
Neurogenesis
Insulin-like growth factor
IGF-1
Aging
Microglia

ABSTRACT

Aging is associated with low-grade neuroinflammation including primed microglia that may contribute to deficits in neural plasticity and cognitive function. The current study evaluated whether exercise modulates division and/or activation state of microglia in the dentate gyrus of the hippocampus, as activated microglia can express a classic inflammatory or an alternative neuroprotective phenotype. We also assessed hippocampal neurogenesis to determine whether changes in microglia were associated with new neuron survival. Adult (3.5 months) and aged (18 months) male BALB/c mice were individually housed with or without running wheels for 8 weeks. Mice received bromodeoxyuridine injections during the first or last 10 days of the experiment to label dividing cells. Immunofluorescence was conducted to measure microglia division, co-expression of the neuroprotective indicator insulin-like growth factor (IGF-1), and new neuron survival. The proportion of new microglia was increased in aged mice, and decreased from wheel running. Running increased the proportion of microglia expressing IGF-1 suggesting exercise shifts microglia phenotype towards neuroprotection. Additionally, running enhanced survival of new neurons in both age groups. Findings suggest that wheel running may attenuate microglia division and promote a proneurogenic phenotype in aged mice.

1. Introduction

Microglia are a highly plastic group of immune cells that reside in the central nervous system (CNS). In the absence of an infection or injury microglia maintain a resting phenotype, in which the cells survey the environment for potential threats. If a threat is detected, microglia undergo morphological changes, proliferate, and begin to release cytoactive agents including proinflammatory cytokines [e.g., interleukin-1β (IL-1β) and IL-6] (Colton, 2009). The expression of this classic inflammatory phenotype facilitates recovery from an insult. Microglia can also express a neuroprotective "alternative" phenotype that participates in regenerative processes, cell growth, and inhibition of inflammation (Colton, 2009). In their alternative state, microglia can produce growth factors, such as insulin-like growth factor (IGF-1) and brain derived neural growth factor (BDNF) (Nakajima et al., 2002; Wine et al., 2009), as well as anti-inflammatory cytokines. Signals from the environment likely determine whether microglia remain in a resting state or undergo activation to either the classic inflammatory or alternative neuroprotective phenotype.

Normal aging alters microglia activity, as microglia in the brain of uninjured aged animals are shifted towards the inflammatory (i.e., classic) phenotype (Dilger and Johnson, 2008). There is a basal increase in proinflammatory cytokine production and increased expression of surface markers (e.g., MHC II and CD86) that are associated with classic microglia activation (Dilger and Johnson, 2008; Frank et al., 2006; Sierra et al., 2007). The age-related priming of microglia contributes to a prolonged neuroinflammatory response following an immune challenge that results in exaggerated expression of sickness behaviors and cognitive deficits compared to adult animals (Dilger and Johnson, 2008; Godbout et al., 2005; Kohman et al., 2007). Another component of the age-related alterations in microglia is an increase in proliferation. Prior reports have shown that microglia from aged animals show increased proliferation in culture as well as *in vivo* in the retina (Inman and Horner, 2007; Rozovsky et al., 1998), but the relevance of increased microglia division to their activation status and age-related changes in neural function has yet to be investigated.

One area of the brain important for cognitive performance and sensitive to aging is the hippocampus. The hippocampus is also of interest because it is one of two areas of the brain that unarguably retains the ability to generate new neurons beyond early development. These new neurons are suggested to participate in learning and memory processes (Clark et al., 2008; Deng et al., 2010; Shors et al., 2002; van Praag et al., 2005). Microglia play a complex role in

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doi:10.1016/j.bbi.2011.10.006

regulating hippocampal neurogenesis, as depending on their state of activation they can facilitate or inhibit neurogenesis (Yirmiya and Goshen, 2011). For example, administration of the endotoxin lipopolysaccharide (LPS) reduces hippocampal neurogenesis and this effect is mediated by microglia, as administration of the microglia inhibitor, minocycline, blocks the reduction in neurogenesis (Ehninger et al., 2011). In contrast, microglia in the resting or alternative state support hippocampal neurogenesis, as microglia participate in new cell migration, increase the number of cells that differentiate into neurons and express molecules (e.g., IGF-1) that can enhance neurogenesis (Aarum et al., 2003; Trejo et al., 2008; Walton et al., 2006; Ziv et al., 2006).

The age-related changes in microglia and proinflammatory cytokine activity are reported to further reduce hippocampal neurogenesis. For example, Gemma et al. (2007) report that administration of a capase-1 inhibitor, that would decrease conversion of pro-IL-1β to the mature form, significantly increased hippocampal neurogenesis in aged, but not young mice. Additionally, administration of the anti-inflammatory chemokine fractalkine (i.e., CX3CL1 or neurotactin), which helps maintain microglia in a resting state, increased hippocampal neurogenesis in aged, but not young, animals (Bachstetter et al., 2009). Collectively, these data indicate that attenuating age-related changes in microglia activity may enhance new neuron survival.

One intervention known to improve cognition and health, particularly in aged individuals, is exercise. Additionally, exercise has immunomodulatory effects that have been well characterized within the peripheral nervous system (Woods et al., 2009). However, the ability of exercise to modulate immune activity within the CNS has not been as thoroughly investigated. The existing literature indicates that exercise has direct effects on microglial cell activity. For example, treadmill running was reported to attenuate the age-dependent increase in microglia activation, as measured by staining intensity of CD11b, in a transgenic mouse model of Alzheimer's disease (Nichol et al., 2008). A recent study found that wheel running attenuated LPS-induced IL-1β production from microglia isolated from aged rats compared to aged sedentary rats (Barrientos et al., 2011). Additionally, wheel running prevented *Escherichia coli*-induced reductions in BDNF expression in the hippocampus following learning, indicating that running offers some protection against negative effects of immune activation (Barrientos et al., 2011). Further, running has been reported to decrease microglia proliferation in the septum and amygdala of adult mice (Ang et al., 2004; Ehninger et al., 2011). Whether exercise modulates microglia proliferation in the aged is unknown.

The objective of the current study was to determine whether normal aging increases microglia division within the hippocampus and whether voluntary wheel running would alter the age-related changes in microglia division. Additionally, we assessed the possibility that exercise increases production of IGF-1 from microglia, which may indicate shifting towards the alternative phenotype. Microglia have been shown to promote regeneration through the release of BDNF and IGF-1 (Nakajima et al., 2002; Wine et al., 2009). Lastly, we assessed whether running-induced changes in microglia activity were associated with enhancements in hippocampal neurogenesis.

2. Methods

2.1. Experimental subjects

Subjects were 32 adult (3.5-month-old) and 31 aged (18-month-old) male BALB/c mice from an in-house aging colony. Mice were given *ad libitum* access to food and water and housed under a reverse 12 h light/dark cycle in the AAALAC approved animal facility. Prior to the start of the experiment mice were group housed (3–4 mice per cage), but during the experiment mice were individually housed. Animals were treated in compliance with the Guide for the Care and Use of Laboratory Animals and the experiment was conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Illinois at Urbana-Champaign.

2.2. Experimental design

Mice were divided by age into either the exercise (access to a running wheel) or sedentary condition for 8 weeks. Half of the sedentary and exercise mice received daily intraperitoneal (i.p.) injections of bromodeoxyuridine (BrdU: 50 mg/kg), a thymidine analogue that incorporates into dividing cells, during the initial 10 days (BrdU Early) of the study while the others received i.p. injections of saline. The remaining mice received i.p. BrdU injections during the last 10 days (BrdU Late) prior to tissue collection while mice given BrdU earlier received saline injections (all mice had a total of 20 i.p. injections). There was a total of eight treatment groups (Age × Exercise condition × BrdU timing). Mice in the sedentary condition were individually housed in standard polypropylene shoebox cages (29 cm L × 19 cm W × 13 cm H). Mice in the exercise condition were individually housed in cages (36 cm L × 20 cm W × 14 cm H) with a 23 cm diameter running wheel (Respironics, Bend, OR). Its important to note that individually housing animals can affect measures of neuroinflammation as well as hippocampal neurogenesis (Ben Menachem-Zidon et al., 2008; Ibi et al., 2008). However, individually housing mice was necessary to prevent competition for access to the running wheel and to monitor running behavior in individual mice, as well as to eliminate the influence of social hierarchy and fighting which can also be a form of stress (Kinsey et al., 2008). Throughout the 8 weeks, wheel rotations were continuously collected in 1 min intervals via magnetic switches interfaced to a computer using the VitalView software (Respironics, Bend, OR). Sedentary mice were deliberately not housed in cages with locked wheels since mice climb in locked wheels (Koteja et al., 1999; Rhodes et al., 2000, 2003) and we wanted to limit physical activity in the sedentary group. Animals were weighed weekly, throughout the experiment.

2.3. Immunohistochemistry

2.3.1. Perfusions

Following the 8 weeks of wheel running or sedentary housing all mice were transcardially perfused with 4% paraformaldehyde. Perfusions occurred 24 h after the final BrdU injection for mice receiving BrdU during the last 10 days of the study. Brains were fixed overnight in 4% paraformaldehyde and then transferred into 30% sucrose solution. Brains were sectioned at 40 micrometers on a cryostat.

2.3.2. BrdU-DAB

A one-in-six series was stained for BrdU to identify newly divided cells. Briefly, free floating sections were rinsed in tissue buffering solution (TBS) and then treated with 0.6% hydrogen peroxide for 30 min. To denature DNA, sections were placed in a solution of 50% de-ionized formamide for 120 min at 65 °C, followed by 10% 20× SCC buffer for 15 min, then 2 N hydrochloric acid for 30 min at 37 °C, and then 0.1 M boric acid (pH 8.5) for 10 min. Sections were blocked with a solution of 0.3% Triton-X and 3% goat serum in TBS (TBS-X plus) for 30 min, and then incubated with the primary antibody rat anti-BrdU (1:200; AbD Serotec, Raleigh, NC, USA) in TBS-X plus for 72 h at 4 °C. After washing with TBS, sections were treated with TBS-X plus for 30 min and then incubated

with a biotinylated goat anti-rat secondary antibody (1:250) in TBS-X plus for 100 min at room temperature. Sections were then treated with the ABC system (Vector, Burlingame, CA, USA) and stained using a diaminobenzidine kit as the chromogen (DAB; Sigma, St. Louis, MO, USA).

2.3.3. Triple labeled immunofluorescence

Separate one-in-six series were triple-labeled with either rat anti-BrdU (1:200), rabbit anti-Iba-1 (1:500; Wako chemicals, Richmond, VA), and goat anti-IGF-1 (1:50, Santa Cruz Biotechnology, Santa Cruz, CA) or rat anti-BrdU, mouse anti-neuronal nuclear protein (NeuN; 1:50; Millipore, Billerica, MA, USA), and rabbit anti-Iba-1 (1:500). Free-floating sections were handled as described above with the exception of the use of a cocktail of primary antibodies. Fluorescent markers (Cy2, Cy3, and Cy5) were conjugated to secondary antibodies, made in goat or donkey, at a dilution of 1:200 and were also delivered as a cocktail. ABC and DAB steps were omitted.

2.4. Image analysis

2.4.1. Hippocampal neurogenesis

Number of new neurons per cubic millimeter granule layer per mouse was calculated as number of BrdU positive cells per cubic millimeter granule layer multiplied by the average proportion of BrdU positive cells that co-expressed NeuN for a given experimental group. To obtain these estimates, the entire granule layer (bilateral), from the one-in-six series stained for BrdU-DAB, was imaged by systematically advancing the field of view of a Zeiss brightfield light microscope, and taking multiple photographs, via Axiocam interfaced to a computer, under 10× (total 100×) magnification. Images were analyzed by ImageJ software. For each image, the granule layer was outlined, and BrdU-positive nuclei were automatically counted by setting a fixed threshold to remove the background. The threshold selected was validated by comparing automated counts to hands counts. In addition, the area (pixels) within the trace was recorded. Estimates of total number of BrdU positive cells are expressed 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 unbiased estimates. These values were then multiplied by the percentage of BrdU cells expressing NeuN estimated from a separate confocal analysis. A confocal Leica SP2 laser scanning confocal microscope (using a 40× oil objective, pinhole size 81.35 μm) was used to determine the average group proportion of BrdU positive cells that differentiated into neurons as indicated by co-expression of NeuN in the granule cell layer of the dentate gyrus. This proportion was based on a minimum of 40 BrdU+ cells per group.

2.4.2. Microglia proliferation and phenotype

A confocal microscope was used to identify microglia (Iba-1+) that divided (Iba-1+ and BrdU+) in the dentate gyrus proper. The percentage of Iba-1+ cells that displayed BrdU was calculated by dividing the number of cells that co-labeled with Iba-1 and BrdU by the number of Iba-1 positive cells analyzed and multiplied by 100. On average 210 Iba-1 positive cells were analyzed within the dentate gyrus per animal. Additionally, we determined the percentage of Iba-1 positive cells in the dentate gyrus that co-labeled with the growth factor IGF-1 by dividing the number of Iba-1 positive cells that displayed IGF-1 by the number of Iba-1 positive cells sampled and multiplied by 100. To determine the proportion of recently divided microglia (Iba-1+ and BrdU+) that displayed IGF-1 we divided the number of triple-labeled cells (Iba-1+, BrdU+, and IGF-1+) by the number of Iba-1 positive cells and multiplied by 100.

2.5. Statistical analysis

Body weight was analyzed by repeated measures ANOVA with Age, Exercise condition, and BrdU timing as the between-subjects variables and Day as the within-subjects (i.e., repeated-measure) variable. Distance run was analyzed by repeated measures ANOVA with Age as the between-subjects variable and Day as the within-subjects variable. BrdU-DAB and the number of new neuron data were analyzed by ANOVA with Age, Exercise condition, and BrdU timing as the between-subjects variables. Neuronal differentiation (proportion of BrdU positive cells expressing NeuN), microglia division (proportion of Iba-positive cells expressing BrdU), and IGF expression (proportion of Iba-positive cells expressing IGF-1) data were analyzed by logistic regression. For these analyses, the deviance is reported in place of the F statistic. An alpha level of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Body weight

As expected, aged mice weighed more than adult mice ($F(1,61) = 407.88$; $p < 0.0001$; data not shown). There was also a significant Exercise condition × Day interaction ($F(7427) = 3.35$; $p < 0.01$; data not shown), that showed mice with access to running wheels weighed less than sedentary mice during the initial weeks, but did not differ from sedentary mice during the later weeks of the study.

3.2. Wheel running

Overall aged mice ran significantly less than adult mice as shown by a significant main effect of Age and an Age × Day interaction ($F(1,26) = 48.36$; $p < 0.0001$; $F(56,1456) = 5.29$; $p < 0.0001$, respectively; see Fig. 1). Aged mice ran an average of 4.75 km/day and adult mice ran an average of 7.20 km/day. Additionally, there was a significant main effect of Day for distance (km) run per day ($F(56,1456) = 30.64$; $p < 0.0001$). Fig. 1 shows that distance run tended to increase daily across both age groups during the first month and then slightly declined the last week.

3.3. Hippocampal neurogenesis

3.3.1. Neuron differentiation

Table 1 shows the proportion of BrdU + cells that co-labeled with NeuN within each experimental group. Aged mice had a lower proportion of new cells expressing NeuN compared to adult mice (deviance = 67.09, $p < 0.001$). Wheel running increased the

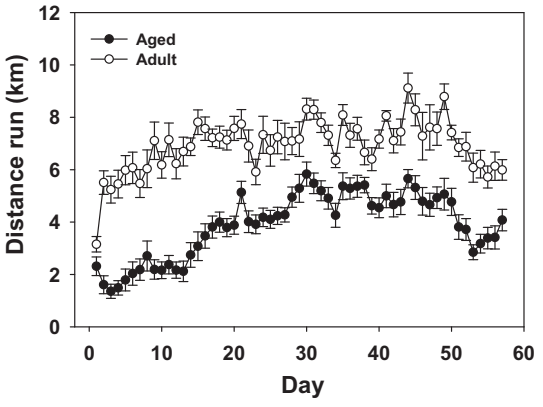

Fig. 1. Average distance (km) run per day by aged and adult mice over eight weeks of running wheel access. Means ± standard error of the mean (SEM).

Table 1
Estimated number of BrdU positive cells in the granular cell layer of the hippocampus per cubic millimeter. The BrdU cell numbers were compared between groups using ANOVA. The proportion of BrdU positive cells that differentiated into new neurons (BrdU+ and NeuN+ cells) was determined by counting a minimum of 40 BrdU positive cells within each of the eight experimental groups and determining the proportion of those cells that co-labeled with NeuN to get an average group proportion. Logistic regression was used to compare the group proportions.

Experimental group	BrdU positive cells in granule layer × 103/mm^3 (SEM)	Percentage of BrdU+ cells displaying NeuN (SEM)
Adult runner early	12.94 (0.60)*	89.9 (0.01) *
Adult sedentary early	4.18 (0.15)	77.8 (0.02)
Adult runner late	5.35 (0.78)	73.4 (0.04)
Adult sedentary late	3.75(0.10)	74.3 (0.04)
Aged runner early	1.31 (0.28)+	70.6 (0.03)+,*
Aged sedentary early	1.16 (0.55)+	57.0 (0.04)+
Aged runner late	2.43 (0.10)+,*	70.7 (0.07)*
Aged sedentary late	1.08 (0.05)+	56.3 (0.06)+

* Significant difference from age-matched sedentary mice.
+ Significant difference between adult and aged mice within an exercise condition.

proportion of BrdU positive cells that displayed NeuN in all groups except in adults when BrdU was administered the last 10 days of the experiment (BrdU Late). This was reflected in a significant main effect of Exercise condition (deviance = 26.13; $p < 0.001$), BrdU timing (deviance = 4.32, $p < 0.05$) and Exercise condition × BrdU timing interaction (deviance = 4.09,4 $p < 0.05$).

3.3.2. Number of new neurons

Aged mice showed a reduction in the number of new neurons (i.e., total number of BrdU cells in the granule layer multiplied by the fraction displaying NeuN) as compared to adult mice ($F(1,55) = 245.77$; $p < 0.0001$; see Fig. 2). Wheel running significantly increased number of new neurons as compared to sedentary in all groups except aged animals given BrdU injections during the initial 10 days (BrdU Early). This was reflected in a significant effect of Exercise condition ($F(1,55) = 112.83$; $p < 0.0001$), BrdU timing ($F(1,55) = 55.06$; $p < 0.0001$), and three-way interaction between Exercise condition, BrdU timing and Age $F(1,55) = 46.95$; $p < 0.0001$).

3.4. Microglia division and phenotype

3.4.1. Microglia division

Aged mice had a significantly higher proportion of Iba-1 positive cells that co-labeled with BrdU compared to adult mice. This was reflected in a significant main effect of Age (deviance = 286.40, $p < 0.001$; see Fig. 3). Wheel running reduced the proportion of Iba-1 positive cells that expressed BrdU in the aged, but not the adult mice compared to sedentary controls. This was reflected in a significant main effect of Exercise condition and an Age × Exercise interaction (deviance = 8.94, $p < 0.01$; deviance = 8.53, $p < 0.01$, respectively; see Fig. 3).

Aged mice given BrdU injections during the initial 10 days of wheel access (Early group) showed a greater proportion of Iba-1 positive cells expressing BrdU than aged mice that received BrdU injections during the last 10 days of the study (Late group); no difference was observed between the adult mice given BrdU during the initial or last 10 days of the study. This was indicated by a significant main effect of BrdU timing and an Age × BrdU timing interaction (deviance = 11.43, $p < 0.001$; deviance = 17.53, $p < 0.001$, respectively; see Fig. 3).

3.4.2. IGF-1 positive microglia

Wheel running significantly increased the proportion of Iba-1 positive cells that co-expressed IGF-1 in both aged and adult mice. This was reflected in significant main effects of Exercise condition (deviance = 160.25, $p < 0.001$; see Fig. 4B). A significant main of Age (deviance = 241.61, $p < 0.001$; see Fig. 4B) showed that aged mice had a higher proportion of Iba-1 positive cells that displayed IGF-1 compared to adult mice. The interaction between age and exercise condition was not significant.

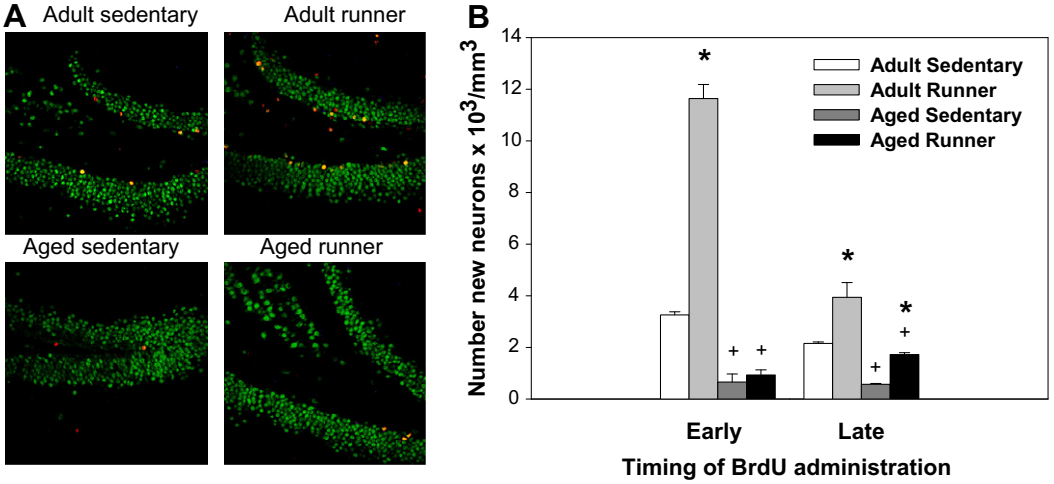

Fig. 2. (A) Representative sections triple labeled with antibodies against Iba-1 (macrophage/microglia; blue), NeuN (mature neuron; green), and BrdU (new cell; red) from adult and aged mice injected with BrdU during the first ten days (BrdU Early). (B) Number of new neurons in the granular cell layer of the hippocampus of adult and aged mice housed with or without running wheel access. The number of new neurons was assessed in cells labeled with BrdU during the first (BrdU Early) or last (BrdU Late) 10 days of the study. * indicates a significant difference from age-matched sedentary mice. + indicates a significant difference between adult and aged mice within an exercise condition. Means ± SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

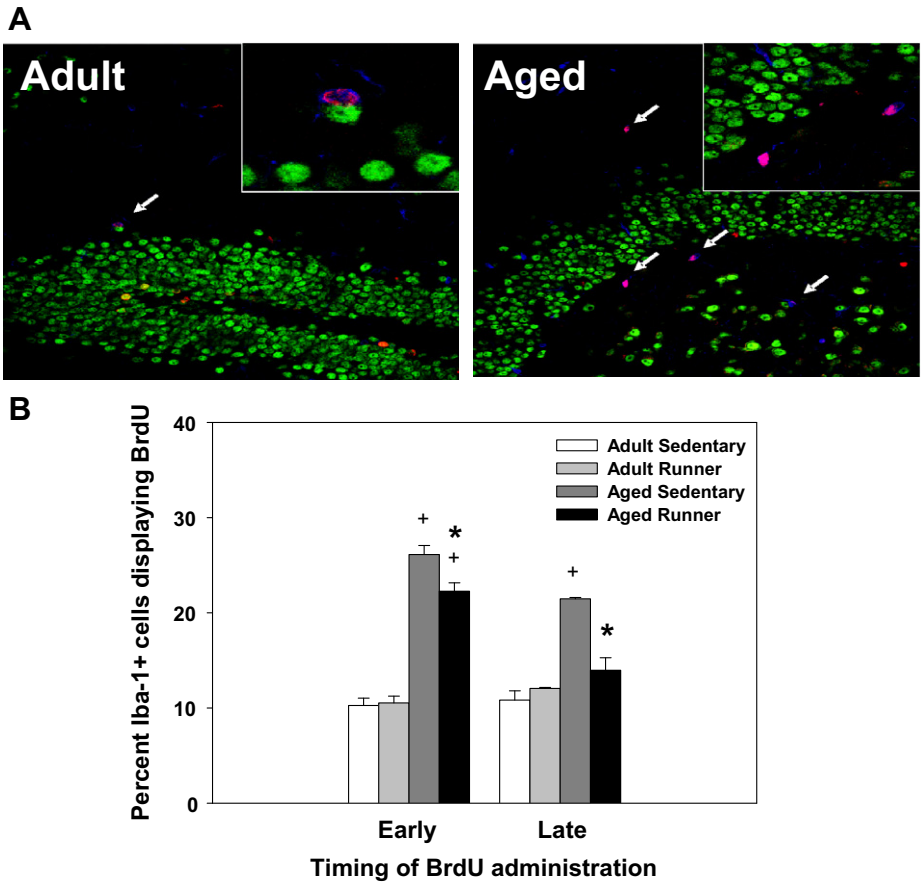

Fig. 3. (A) Representative hippocampal sections triple labeled with antibodies against Iba-1 (macrophage/microglia; blue), NeuN (mature neuron; green), and BrdU (new cell; red) from adult and aged mice. (B) Average percentage of microglia that underwent division in the dentate gyrus of adult and aged mice. Percentages were calculated by dividing the number of Iba-1 positive cells that expressed BrdU by the number of Iba-1 positive cells and multiplying by 100. ∗ indicates a significant difference from age-matched sedentary mice. + indicates a significant difference between adult and aged mice within an exercise condition. Means ± SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Wheel running significantly increased the proportion of new microglia (Iba-1+ and BrdU+) that expressed IGF-1 compared to sedentary mice. This was reflected in a significant main effect of Exercise condition (deviance = 123.84, $p < 0.001$; see Fig. 4D). Additionally, aged mice showed a higher proportion of new microglia that displayed IGF-1 than adult mice. This was reflected in a significant main effect of Age (deviance = 49.72, $p < 0.001$; see Fig. 4D). Runners that received late BrdU injections (last 10 days) showed a higher proportion of new microglia that expressed IGF-1 than runners given early BrdU injections (first 10 days), which was particularly evident in the aged mice (see Fig. 4D). This was reflected in a significant Age × Exercise condition interaction (deviance = 5.31, $p < 0.05$) and a significant Exercise condition × BrdU timing interaction (deviance = 4.15, $p < 0.05$).

4. Discussion

Exercise is a valuable intervention to delay or reverse age-related alterations in cognitive decline and disease susceptibility including Alzheimer's and Parkinson's disease (Kramer et al., 2006). Recent evidence indicates that exercise may have direct effects on microglia, as wheel running attenuates microglia cytokine production (Barrientos et al., 2011). The results of our studies extend the existing literature by demonstrating that aging increases the proportion of new microglia in the hippocampus. Further, we report that wheel running reduces the proportion of new microglia in aged mice and increases their expression of a proneurogenic phenotype that may contribute to the running-induced increase in hippocampal neurogenesis. Collectively, the results provide novel evidence that wheel running may combat age-related alterations in microglia and increase their expression of IGF-1 a phenotype conducive for neuroprotection, growth and brain plasticity.

Aging is associated with priming of microglia towards the inflammatory phenotype, as shown by increased expression of MHC II (Frank et al., 2006; Sierra et al., 2007). Our data extend this observation by revealing that microglia in the hippocampus show an age-related enhancement in division relative to adult mice. This increase in the proportion of new microglia (i.e., BrdU+ and Iba-1+ cells) may result from an increase in both the survival and proliferation of microglia, as aged mice had greater proportions of Iba-1 positive cells displaying BrdU when the cells were labeled either 2 months or 10 days prior to tissue collection. Our findings are consistent with prior *in vitro* work, as microglia collected from the cortices of aged rats showed increased proliferation in culture compared to young rats (Rozovsky et al., 1998). Similarly, Inman and Horner (2007) report that a significantly greater proportion of new microglia (BrdU+ and Iba-1+) were present in the retina of middle-aged mice compared to young mice. In conjunction with our findings, the work indicates that aging increases microglia division in various regions of the brain. The mechanisms of the age-related increase in microglia division have yet to be fully elucidated. However, in culture, microglia from aged rats showed decreased responsiveness to the anti-inflammatory cytokine

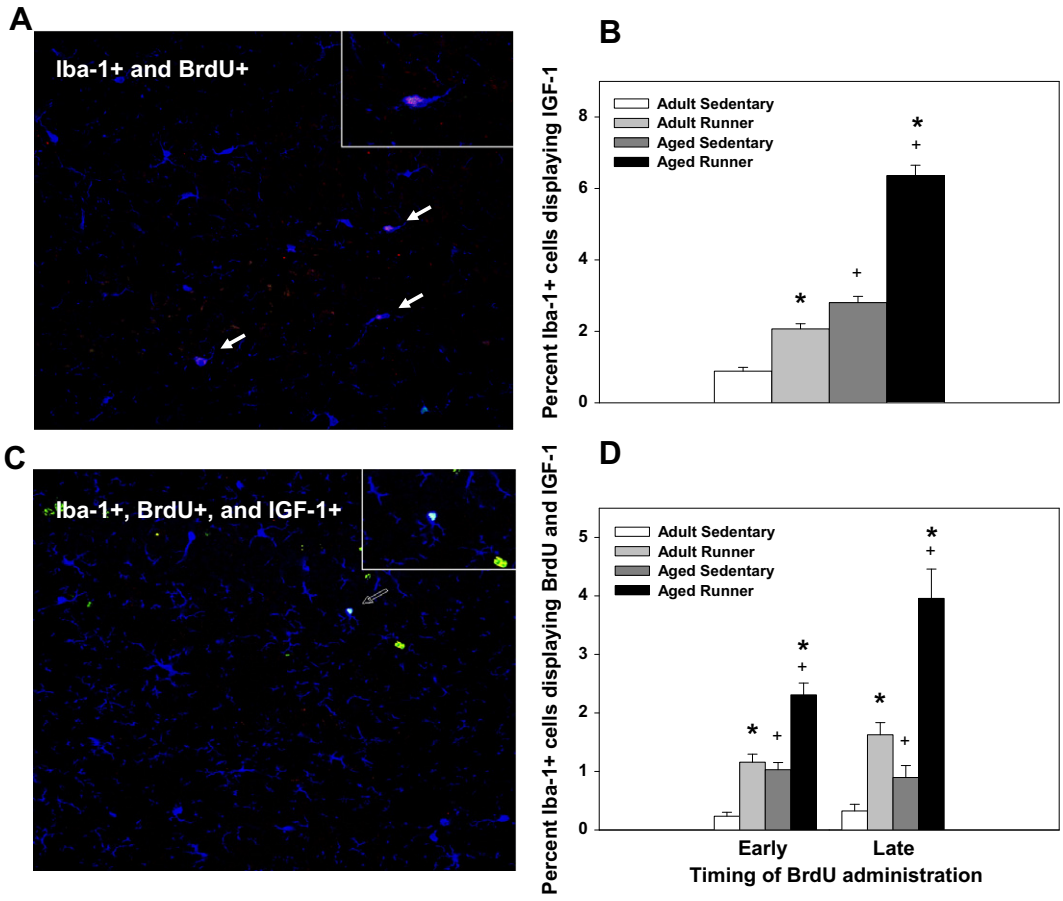

Fig. 4. (A) Representative image of a cell co-labeled with antibodies against Iba-1 (macrophage/microglia; blue) and IGF-1 (growth factor; red) from an aged mouse. (B) Average percentage of Iba-1 positive cells that expressed IGF-1 in the dentate gyrus of adult and aged mice. Data are collapsed across BrdU timing. (C) Representative image of a triple-labeled cell with antibodies against Iba-1 (macrophage/microglia; blue), IGF-1 (growth factor; red), and BrdU (new cell; green) from an aged mouse. (D) Average percentage of Iba-1 positive cells that are new (BrdU+) and express IGF-1. ∗ indicates a significant difference from age-matched sedentary mice. + indicates a significant difference between adult and aged mice within an exercise condition. Means ± SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

transforming growth factor-β (TGF-β) that normally inhibits microglia proliferation (Rozovsky et al., 1998) potentially indicating that reduced regulatory control may contribute to the enhanced microglia division.

The observed increase in the proportion of dividing microglia in the aged versus adult mice raises the question of whether the total number of microglia is increased in aged mice. Though not assessed in the current study, a few reports have evaluated whether aging increases the number of microglia within the hippocampus by stereology. The initial report found no significant difference in the total number of microglia labeled with Mac-1 in adult (4 month) compared to aged (27 month) male C57BL/6 J mice, as less than a 10% increase in microglia was observed in the aged mice (Long et al., 1998). However, a separate study that used female C57BL/6 J mice, conducted by the same laboratory, found a significant age-related increase in the number of microglia in the dentate gyrus and CA1 region of the hippocampus compared to adult females (Mouton et al., 2002). Collectively these data indicate a sex difference exists, but whether a strain differences also exists has yet to be assessed. Alternatively, the increase in microglia division we observed in the aged male BALB/c mice may be accompanied by an increase in cell turnover; thereby the total number of microglia would remain similar to the adults. Additional work is needed to clarify these possibilities.

Prior work has shown that exercise influences microglia activity, including microglia proliferation. For instance, exercise decreased microglia proliferation in the septum and amygdala in adult animals (Ang et al., 2004; Ehninger et al., 2011). However, Ehninger and Kempermann (2003) report that exercise increased microglia proliferation in the cingulate and motor cortex of adult subjects. In contrast to these findings we found no effect of wheel running on microglia survival or proliferation in the dentate gyrus in the adult mice. We observed similar proportions of BrdU positive microglia in the runner and sedentary adult mice. These differences likely relate to procedural differences (i.e., type and duration of exercise) as well as the brain region assessed.

Our data show that exercise reduces the proportion of microglia in the dentate gyrus that divide in aged mice. This reduction in the proportion of new microglia appears to result from a decrease in both cell survival as well as proliferation. However, wheel running had a greater impact on proliferation, as we observed a larger reduction in the proportion of new microglia when cell were labeled just prior to tissue collection (BrdU Late; see Fig. 3B). Whether other brain regions would show a similar reduction following exercise is unknown, as regional variations are reported in young animals (Ang et al., 2004; Ehninger and Kempermann, 2003; Ehninger et al., 2011). Given the involvement of the hippocampus in learning and memory, reducing excessive microglia activity may be of particular relevance to cognitive function. Presently, the mechanisms behind the exercise-induced reduction in the proportion of new microglia are unknown, but we can speculate that exercise-induced changes in the microenvironment, such as increasing factors that regulate microglia, may contribute. For instance, Tong et al. (2001) found that wheel running increases

expression of fractalkine, a chemokine released by neurons that regulates microglia activation. Alternatively, exercise may reduce inflammatory molecules that could stimulate microglia proliferation. For example, cultured microglia from aged rats that had access to a running wheel had lower basal (unstimulated) expression of IL-1β and TNF-α compared to microglia from sedentary aged rats (Barrientos et al., 2011). Though more work is needed to identify the mechanism behind these effects, our data provide novel evidence that exercise can attenuate microglia division primarily through reducing microglia proliferation in the aged.

In addition to affecting the proportion of new microglia, our data indicate that wheel running may be capable of altering the phenotype expressed by microglia. Wheel running significantly increased the proportion of microglia that co-labeled with IGF-1 (see Fig. 4B). These findings are consistent with prior research that found microglia increased IGF-1 production following environmental enrichment (that included running wheels) (Ziv et al., 2006). Wheel running significantly increased IGF-1 expression in both new microglia (BrdU+ and Iba-1+) and pre-existing microglia (BrdU- and Iba-1+) in both age groups. These findings indicate that both recently divided and pre-existing microglia are responsive to the changes induced by wheel running. It is interesting that aged mice with access to a running wheel showed a higher percentage of microglia that co-labeled with IGF-1 compared to adult mice. One possibility is that the age-related priming of microglia contributes to this response (Dilger and Johnson, 2008; Frank et al., 2006). Since microglia in aged animals are in a partially active state they may be more responsive to environmental changes whereas microglia in adults typically express the resting phenotype and may have to undergo activation before IGF-1 or other molecules can be expressed.

Increased IGF-1 expression from microglia is proposed to be beneficial to the brain and to promote recovery. For instance, microglia are reported to express IGF-1 following an ischemic injury, which is thought to facilitate survival of cells in the damaged region (O'Donnell et al., 2002). Further, Maher et al. (2006) report that intracerebroventricular administration of IGF-1 to aged rats significantly attenuated the interferon-gamma (IFN-γ) induced microglia activation and subsequent IL-1β expression. Collectively, findings indicate that increased IGF-1 levels may help reduce neuroinflammation and facilitate recovery and cell survival. Interestingly, we found an age-related increase in the proportion of Iba-1 positive cells that display IGF-1. This increase in the aged may be a compensatory response to other age related changes occurring within the hippocampus such as an increase in oxidative stress (Piriz et al., 2010) or increased IGF resistance that is related to reductions in IGF receptor expression (Broughton and Partridge, 2009). However, there is some evidence that increased IGF expression can impair spatial learning ability and increase production of free radicals (Broughton and Partridge, 2009). Further work is needed to elucidate whether the age-related proportional increase in IGF expression from microglia is neuroprotective or detrimental to hippocampal function.

Aging is associated with a substantial decline in hippocampal neurogenesis. Consistent with prior reports (van Praag et al., 2005; Walter et al., 2011), we observed a significant reduction in both the proliferation and rate of survival of new cells in aged mice compared to adults. Additionally, fewer new cells differentiated into neurons in the aged (approximately 57%) compared to adults (approximately 77%) under sedentary conditions (see Table 1). In both age groups, wheel running increased hippocampal neurogenesis. Confirming prior reports (Clark et al., 2008; van Praag et al., 2005), we observed a significant increase in the number of new cells and increased neuron differentiation in both age groups following wheel running.

Microglia have been reported to contribute to the age-related decrease in hippocampal neurogenesis, as inhibition of microglia activation or IL-1β is known to increase neurogenesis in aged, but not young, animals (Bachstetter et al., 2009; Gemma et al., 2007). One possibility is that the increased proportion of microglia expressing of IGF-1 may contribute to the exercise-induced increase in hippocampal neurogenesis, particularly in the aged mice. Prior work suggests that microglia, through expression of IGF-1, may play a role in supporting enhanced neurogenesis following injury (Thored et al., 2009). Through not assessed in the current study, prior work has shown that exercise increases levels of IGF-1 and BDNF within the brain (Nakajima et al., 2010; Stranahan et al., 2009). Therefore other growth factors as well as cell types likely contribute to the exercise-induced increase in neurogenesis. Though additional work is needed to determine the specific contribution of IGF-1 derived from microglia to hippocampal neurogenesis, the present data indicate that exercise increases the proportion of microglia that express a proneurogenic phenotype.

It is important to note that the current study used Iba-1 that labels both microglia and macrophages. Therefore, it is possible that a portion of the cells we identified were macrophages. Though the origin of microglia remains controversial, a recent paper proposed that microglia originate from yolk sac macrophages during early embryonic development (Ginhoux et al., 2010). Further, the authors propose that perinatal hematopoietic cells, including monocytes, have minimal contribution to maintaining the adult microglia population, as experiments that employed parabiosis or transplantation of donor bone marrow cells revealed a minimal number of donors cells entered the brain (Ginhoux et al., 2010). These data indicate that microglia precursors that localize in the brain during embryonic development may be primarily responsible for maintaining the microglia population throughout adulthood; potentially indicating that majority of the Iba-1+ cells we identified were microglia. Though others have reported that microglia proliferation and macrophage infiltration may have equal contributions to maintaining the steady-state levels of microglia (Lawson et al., 1992). Whether the relative contributions of macrophage infiltration and microglia proliferation to homeostatic levels of microglia changes across the lifespan is currently unknown.

The age-related priming of microglia towards the inflammatory phenotype is a central factor in increasing susceptibility to inflammation-related behavioral and cognitive deficits and possibly the progression of neurodegenerative diseases. Our data indicate that exercise may be capable of enhancing features of the alternative neuroprotective phenotype, by increasing the proportion of microglia that produce IGF-1. Further we report that exercise attenuates the age-related increase in microglia division primarily through reducing microglia proliferation that in combination with increased IGF-1 expression may aid in reducing neuroinflammation and subsequently enhance hippocampal neurogenesis and potentially cognitive function. Additional work is needed to determine whether exercise enhances microglia expression of other neuroprotective factors such as BDNF or nerve growth factor (NGF) and whether these changes are associated with improvements in cognitive performance.

Funding

This work was supported by grants from National Institute of Health, MH083807 and DA027487 to J.S.R and from National Institute on Aging K99AG0404184 to R.A.K. Funding sources had no involvement in the experimental design or interpretation of the results.

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