VOLUNTARY EXERCISE ALTERS THE CYTO-ARCHITECTURE OF THE ADULT DENTATE GYRUS BY INCREASING CELLULAR PROLIFERATION, DENDRITIC COMPLEXITY, AND SPINE DENSITY

by

BRENNAN DANIEL EADIE

B.A., University of British Columbia, 2002

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES
(Graduate Programme in Neuroscience)

We accept this thesis as conforming
to the required standard

.....
Dr. Brian R. Christie (Supervisor)

.....
Dr. Lynn A. Raymond (Committee Head)

.....
Dr. Anthony G. Phillips (Committee Member)

.....
Dr. Yu Tian Wang (External)

THE UNIVERSITY OF BRITISH COLUMBIA

August 2004

© Brennan Daniel Eadie, 2004

Library Authorization

In presenting this thesis in partial fulfillment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Brennan Daniel Eadie
Name of Author *(please print)*

12/08/04
Date (dd/mm/yyyy)

Title of Thesis: Voluntary exercise alters the cyto-architecture of the adult dentate gyrus by increasing cellular proliferation, dendritic complexity, and spine density

Degree: Master of Science Year: 2004

Department of Neuroscience
The University of British Columbia
Vancouver, BC Canada

Abstract

While a dramatic increase in the number of bromodeoxyuridine (BrdU)-positive cells in the adult dentate gyrus following voluntary exercise has been consistently reported, it has never been determined whether this reflects an increase in neurogenic activity or an exercise-induced increase in the metabolic processing of systemically injected BrdU (a compound that inserts itself into the DNA of cells undergoing replication in preparation for cell division). In these experiments, it is shown that (1) 200 mg/kg is a saturating dose of BrdU in both control and voluntary exercise animals, (2) there are almost twice as many BrdU-positive cells following exercise, but only if doses of BrdU of 200 mg/kg or greater are employed, (3) high doses of BrdU (400 and 600 mg/kg) do not affect the appearance or distribution of labeled cells, (4) voluntary exercise leads to similar increases in the number of cells expressing Ki67, an intrinsic marker of cellular proliferation, (5) dendritic length and dendritic complexity are significantly increased in the dentate gyrus regions of animals that exercise, and (6) spine density is significantly greater on the dendrites of dentate granule cells following voluntary exercise. This study demonstrates that exercise up-regulates neurogenic activity in the dentate gyrus region of adult rats, independent of any putative changes in altered BrdU metabolism, and that it substantially alters the morphology of dentate granule cell dendrites. The dramatic changes to the cyto-architecture of the dentate gyrus induced by voluntary exercise may underlie the enhancement of hippocampal long-term potentiation and hippocampal-dependent memory that our group has previously described. These results suggest that exercise may be an effective component of therapeutic regimes aimed at improving the functioning of individuals with neuropathologies that involve the degradation of cells in the hippocampal dentate gyrus.

Table of Contents

List of Tables

List of Figures

Abbreviations

ABC	- avidin-biotin-peroxidase
BBB	- blood-brain-barrier
BDNF	- brain derived neurotrophic factor
BrdU	- bromodeoxyuridine
C_{50}	- control injected with 50 mg/kg BrdU
C_{200}	- control injected with 200 mg/kg BrdU
C_{400}	- control injected with 400 mg/kg BrdU
C_{600}	- control injected with 600 mg/kg BrdU
CA1	- cornu ammon's region 1
CCD	- charge coupled device
CNS	- central nervous system
Con	- control
DAB	- diaminobenzidine
DG	- dentate gyrus
FITC	- fluoroscein
H_2O_2	- hydrogen peroxide
HCl	- hydrochloric acid
i.p.	- intraperitoneal
LTP	- long-term potentiation
NaCl	- sodium chloride
PC	- personal computer
SE	- standard error
SSC	- sodium chloride-sodium Citrate
TBS	- trizma buffered solution
Tris-HCl	- tris hydrochloride
VE	- voluntary exercise
VE_{50}	- voluntary exercise animal injected with 50 mg/kg BrdU
VE_{200}	- voluntary exercise animal injected with 50 mg/kg BrdU
VE_{400}	- voluntary exercise animal injected with 50 mg/kg BrdU
VE_{600}	- voluntary exercise animal injected with 50 mg/kg BrdU
VEGF	- vascular endothelial growth factor

Acknowledgements

I would like to offer Dr. Brian R. Christie my most sincere thanks for his supervision and assistance in the preparation of this thesis. Brian, your patience, wisdom, and positive demeanor make you both an exceptional role model and friend to me. Your guidance has truly been immeasurable. I could not have hoped for a better supervisor.

The many individuals in the laboratory and the Psychology department that I have had the pleasure of working with, that have helped me both theoretically and technically, also deserve a major thank you.

I would like to extend a special thanks to Dr. Raymond and Dr. Phillips for their guidance and critical comments. I have the utmost respect for you both as individuals and scientists.

To my fiancé Rebecca Levi, thank you for your unfailing encouragement and support to achieve my dreams, however bizarre or lofty. You have believed in me from the beginning, giving me the fortitude necessary to start and complete this thesis. You have always been there when I needed you most. I am forever indebted to you for these things. I love you.

Last, but far from least, I would like to thank my family. I know that all of you are still pleasantly surprised that I chose an academic pursuit over my dreams of becoming a professional skateboarder. I told you that I didn't have to do my homework! You have inspired me to open my mind to the world and shown me how to stay grounded at the same time. I love you all and appreciate your support in all of its forms. By the way, I think that skateboarding pays better.

INTRODUCTION

Recent research has reported that voluntary exercise (VE) increases both cellular proliferation and neurogenesis in the brains of adult mice and rats (Brown et al., 2003; Fabel et al., 2003; Farmer et al., 2004; Holmes, Galea, Mistlberger, & Kempermann, 2004; Kitamura, Mishina, & Sugiyama, 2003; Rhodes et al., 2003; Steiner et al., 2004; van Praag, Christie, Sejnowski, & Gage, 1999; van Praag, Kempermann, & Gage, 1999). In these studies, an optimal/saturating dose was not determined for VE or control animals. Instead, BrdU was administered systemically at a concentration of 50 mg/kg over the course of several days. This injection protocol was employed, in part, because it was originally used in several influential studies examining cell proliferation in developing embryos (Miller & Nowakowski, 1988; Takahashi, Nowakowski, & Caviness, 1992), and because it had been used successfully in studies involving environmental enrichment (Kempermann, Kuhn, & Gage, 1997). Recently, the efficacy of this protocol with adult animals has been questioned, and convincing evidence has been presented demonstrating that a systemic injection of BrdU at a dose of 50 mg/kg is not, in fact, saturating, labeling only a fraction of dividing cells (Cameron & McKay, 2001). This has led to speculation that the increases in BrdU labeling that are observed following VE may actually represent an increase in the efficacy of BrdU to penetrate into the central nervous system and label cells, rather than a change in neurogenic activity per se. Support for this hypothesis could also be drawn from a number of studies showing that exercise also induces angiogenesis (Kleim, Cooper, & VandenBerg, 2002; Swain et al., 2003); increases in cerebral blood volume (Swain et al., 2003); increases in cerebral blood flow (Yancey & Overton, 1993); and transient increases in the permeability of the blood-brain-barrier (BBB) (Sharma, Cervos-Navarro, & Dey, 1991). Thus, it may be that increased activity levels lead to changes in the vasculature resulting in more points of entry for BrdU, and/or BrdU being more efficiently transported across the BBB into the CNS. Interestingly, Cameron and McKay (2001) suggest that the reason that doses of

BrdU greater than 50 mg/kg can be toxic in developing embryos, but not in adult animals, is the development of the BBB shortly after parturition. It is therefore quite plausible that the vasculature impacts the processing of BrdU. One of the major aims of this paper will be to determine whether alterations in neurogenic activity truly occur in the dentate gyrus (DG) following VE.

Recently, researchers have shown that environmental enrichment (which includes exercise), but not exercise alone, changes the length of the dendrites in the hippocampi of group housed mice (Faherty, Kerley, & Smeyne, 2003; Turner & Lewis, 2003). Interestingly, they report that these changes occur in the CA1 region, and not in the dentate gyrus. This is, perhaps, inconsistent with the findings that (1) VE increases levels of brain-derived neurotrophic factor (BDNF) specifically in the dentate gyrus, and (2) BDNF alters cellular and dendritic morphology both *in vitro* and *in vivo* (Ang, Wong, Moochhala, & Ng, 2003; Aoyagi, Nishikawa, Saito, & Abe, 1994; Barde, 1994; Berchtold, Kesslak, Pike, Adlard, & Cotman, 2001; Farmer et al., 2004; McAllister, Katz, & Lo, 1997; McAllister, Lo, & Katz, 1995; Neeper, Gomez-Pinilla, Choi, & Cotman, 1995; Neeper, Gomez-Pinilla, Choi, & Cotman, 1996; Russo-Neustadt, Beard, & Cotman, 1999; Smith & Zigmond, 2003; Tolwani et al., 2002; Tyler & Pozzo-Miller, 2003; Tyler & Pozzo-Miller, 2001). Alterations in dendritic morphology might also explain some of the physiological changes related to long-term potentiation in the DG that our laboratory has observed (Farmer et al., 2004; van Praag, Christie et al., 1999). Modeling studies have demonstrated that even minor alterations in dendritic structure can have a marked impact on the biophysical properties of neurons and dramatically impact the manner in which neurons transmit information (Mainen & Sejnowski, 1995, 1996). For example, young neurons in the adult dentate gyrus are morphologically distinct from other granule neurons, and they exhibit unique electrophysiological properties (Overstreet et al., 2004; Schmidt-Hieber, Jonas, & Bischofberger, 2004; Snyder, Kee, & Wojtowicz, 2001; van Praag et al., 2002). Faherty et al. (2003) group-

housed their animals, and it may be that social factors affected the access to, and/or activity on the running wheel which confounded the interpretation of their results (McKittrick et al., 2000). Here, we employ our standardized exercise protocol (individually housed and monitored), which has been shown to alter BDNF levels, increase long-term potentiation (LTP), and increase neurogenesis, to determine if VE can alter BrdU metabolism and/or dendritic morphology in the DG.

MATERIALS AND METHODS

Adult male Sprague-Dawley rats (200-250g) obtained from the University of British Columbia Animal Care Services were individually housed, maintained on a 12 hour light/dark cycle, and given free access to food and water. After a three day acclimation period, animals (N = 63) were randomly assigned to either standard caging (Control, Con) or caging that contained a running wheel (Voluntary Exercise, VE) connected to a PC computer (Mini-Mitter Systems Inc., WA), allowing us to monitor running frequency, duration and intensity for individual animals. All VE animals used for analyses of cellular proliferation were exposed to a running wheel for a minimum of two weeks and a maximum of three weeks. Two animals were removed from the data because they did not use the running wheel. All VE animals used for analyses of dendritic morphology were exposed to the running wheel for two weeks. Control animals were in their cages for equivalent periods. All of the procedures used in these experiments were in accordance with the Canada Council on Animal Care and approved by the University of British Columbia Animal Care Committee.

Immunohistochemistry

To label proliferating cells, the mitotic marker bromodeoxyuridine (BrdU) was administered as a single i.p. injection at the beginning of the light cycle. BrdU was mixed at a maximum concentration of 20 mg/ml in TBS, and administered at a dose of either 50, 200, 400, or 600 mg/kg, depending upon the experiment. In all experiments, animals were euthenized by Somnotol overdose and then perfused through the heart with 60 ml of 0.9% saline, followed by 60 ml of 4% paraformaldehyde. Brains were removed and stored in paraformaldehyde, for 24 hours before being transferred to 30% sucrose until saturated. Coronal sections (40 μm) were then obtained throughout the extent of the hippocampus using a Leica VT1000 vibratome.

BrdU Staining

Bromodeoxyuridine immunohistochemistry was performed as previously described (van Praag et al., 1999a; Farmer et al., 2004). Briefly, free-floating sections were treated with 0.6% H_2O_2 in Tris-buffered saline (TBS; 0.15 M NaCl, 0.1 M Tris-HCl, pH 7.5) for 30 minutes to block endogenous peroxidase activity. Next, sections were incubated in 50% formamide/2X SSC (0.3 M NaCl, and 0.3 M sodium citrate) at 65 °C, rinsed for 5 minutes in 2X SSC, incubated in 2 N HCl for 30 minutes at 37 °C, and then placed in 0.1 M boric acid (pH 8.5) for 10 minutes to denature the DNA. The tissue was rinsed with TBS several times and subsequently incubated in TBS++ (TBS, 0.1% Triton X-100, and 3% donkey serum). Sections were then incubated with anti-BrdU (Chemicon) diluted at a concentration of 1:100 in TBS++ for 12 hours at 4 °C. After being rinsed with TBS, sections were immersed in biotinylated donkey anti-rat biotin (Chemicon) at a concentration of 1:100 for 4 hours at 4 °C. Following rinses with TBS, avidin-biotin-peroxidase (ABC Elite Kit) was applied for 1 hour, followed by peroxidase detection (ABC DAB kit). Tissue was then mounted and coverslipped for microscopic analysis.

Ki-67 Staining

Ki-67 immunohistochemistry was performed on separate tissue sections that were obtained from the same animals that were used for BrdU histochemistry. Following TBS rinses and brief incubation in TBS++, slices were incubated in a Ki67 antibody (NCL-Ki67-MM1; NovoCastra Labs Ltd.) diluted at a concentration of 1:200 in TBS++ for 48 hours at 4 °C with gentle shaking. The tissue was again rinsed in TBS several times and subsequently incubated briefly in TBS++ (TBS, 0.1% Triton X-100, and 3% donkey serum). After being rinsed with TBS, sections were immersed in biotinylated donkey anti-rat biotin (Chemicon) diluted in TBS at a concentration of 1:1000 for 4 hours at 4 °C. Following rinses with TBS, avidin-biotin-peroxidase (ABC Elite Kit) was applied for 1 hour, followed by peroxidase detection (ABC

DAB kit). Tissue was then mounted and coverslipped. For fluorescence analysis, tissue was incubated in anti-rat FITC (Chemicon) secondary. Tissue was then mounted and covered with Prolong anti-fade (Molecular Probes) and coverslipped. The total number of Ki67-positive cells in the subgranular zone of the dentate gyrus was quantified in a blind fashion using light and fluorescence microscopy.

Golgi Staining

Animals not used for immunohistochemistry were perfused with 0.9% saline (60 ml) and their brains were removed and placed into vials containing 40 ml of modified Golgi-Cox solution (Gibb & Kolb, 1998) and stored in the dark for 14 days. Following this, the brains were switched to a 20% sucrose solution, and stored in the dark for a further 4 days, before being blocked and sectioned at 200 μm. Individual sections were then saved and placed on 2% gelatinized slides and examined under a microscope and analyzed using Image-Pro Plus software (MediaCybernetics, CA). Slices were coded prior to quantitative analysis to ensure that data analysis was conducted in a blind manner. From each brain, ten granule cells from the dorsal blade of the dentate gyrus subfield of the hippocampus were selected based upon the following criteria: (1) the cells were entirely filled and there was dark and consistent impregnation throughout the extent of the dendrites, (2) cells were relatively isolated from neighboring impregnated cells with minimal overlap of filled processes, and (3) the dendrites were completely visible, with no cut processes, and extended the breadth of the dentate molecular layer to the hippocampal fissure while laying in the plane of the section. For analyses of spine density, the number of spines per 10 μm was obtained for 6 segments per cell, and 5 cells per animal. Each neuron was traced using an upright epifluorescence microscope (Olympus BX51), and 40-100X magnification and Image-Pro Plus software (MediaCybernetics, MD). For each neuron, the mean dendritic length was calculated and a Sholl analysis (Sholl, 1956) was

performed to quantify the number of dendritic processes crossing at 15 μm (DG) or 20 μm (CA1) intervals. Dendritic spines were measured from one apical branch in the terminal regions of the dendrites of all cells using a 100X oil immersion lens and Image-Pro Plus software. Digital images were captured using a CoolSNAP-HQ CCD camera. Spine density was calculated from digital images of 10 μm sections of distal dendritic processes. All data were obtained in a blind and consistent manner.

RESULTS

As mentioned above, several studies have reported that VE increases neurogenesis, though none have determined if this effect saturates, as does BrdU labeling, in control animals. This is a critical to determine, as the effects of exercise on the vasculature could increase the metabolic processing of BrdU, and enhance the ability of a sub-saturating dose of BrdU to label cells. This could give the illusion that neurogenic activity is increased in response to VE when, in fact, it is merely the fraction of dividing cells labeled by BrdU that is increased in response to VE. Therefore, our initial focus of interest was to quantify cellular proliferation across a variety of doses of BrdU, in both control and VE animals. We found that the number of BrdU-positive cells reaches asymptotic/saturating levels in control animals at approximately 200 mg/kg. In other words, within the control group, the number of BrdU-positive cells was significantly lower in the subgroup that received an injection of BrdU at a concentration of 50 mg/kg as compared to all other doses employed (200, 400, and 600 mg/kg) (Fig. 1). Control rats that were injected with 50 mg/kg of BrdU exhibited an average of 480 $\pm$143 (SE) newly generated cells in the subgranular zone of the DG, whereas control rats that received 200, 400, and 600 mg/kg of BrdU exhibited 1651$\pm$323, 1296 $\pm$277, and 1776 $\pm$96 respectively ($F_{(3, 13)} = 6.50$, $p<0.05$).

In VE animals, we found that, as with the control animals, asymptotic levels of BrdU labeling in the subgranular zone of the DG occurred at a concentration of BrdU of 200 mg/kg ($F_{(3, 13)} = 6.53$, $p<0.05$). Again, there were no significant differences between the subgroups that received BrdU at concentrations of 200, 400, and 600 mg/kg ($F_{(2, 18)} = 0.78$, $p=0.48$) (Fig. 2). Voluntary exercise animals exhibited an increase in cellular proliferation in the subgranular zone of the DG over controls at all concentrations of BrdU (200 mg/kg, $p<0.05$; 400 mg/kg, $p<0.05$; 600 mg/kg, $p<0.05$) (Table 1), with the exception of the lowest dose which showed a trend towards significancr (50 mg/kg, $p=0.09$). The lack of an increase in the effect size between controls and VE animals at BrdU concentrations of 200 mg/kg and higher suggests that a substantial change

in BrdU metabolism is not responsible for the basic effect of increased ability to label dividing cells in the DG of VE animals.

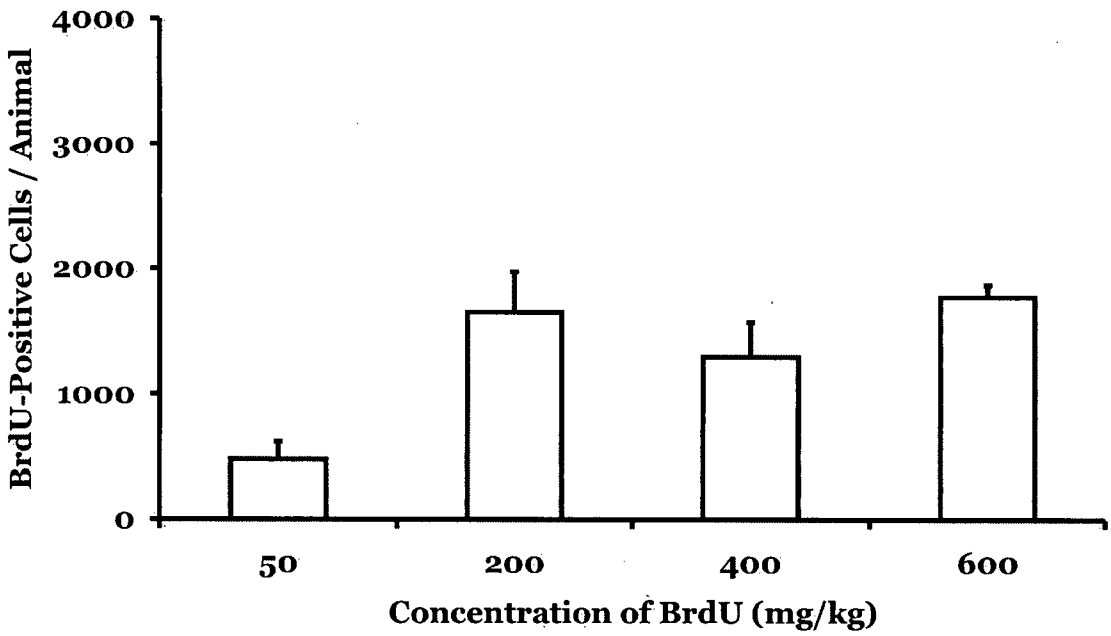

Figure 1. BrdU-positive cells in the subgranular zone of the dentate gyrus 24 hours following a single injection of BrdU at a concentration of either 50, 200, 400, or 600 mg/kg. A saturating dose of BrdU should be used if one wishes to accurately label and estimate the total number of newly generated cells in the adult dentate gyrus. An asymptotic level of BrdU-positive cells was reached with a single i.p. injection of BrdU at a concentration of 200 mg/kg administered 24 hours prior to sacrifice.

Qualitative assessment of the tissue revealed that high concentrations of BrdU (400 and 600 mg/kg) (1) only label cells in the subgranular zone and granule cell layer of the DG in all animals (Fig. 3, left and middle column), and (2) do not lead to a change in the gross appearance of labeled cells (Fig. 3, right column).

As an alternative means to determine whether VE increases cellular proliferation in the DG, we also immuno-stained for Ki67, an intrinsic marker of cellular proliferation (Kee, Sivalingam, Boonstra, & Wojtowicz, 2002). Rats exposed to a running wheel showed more than

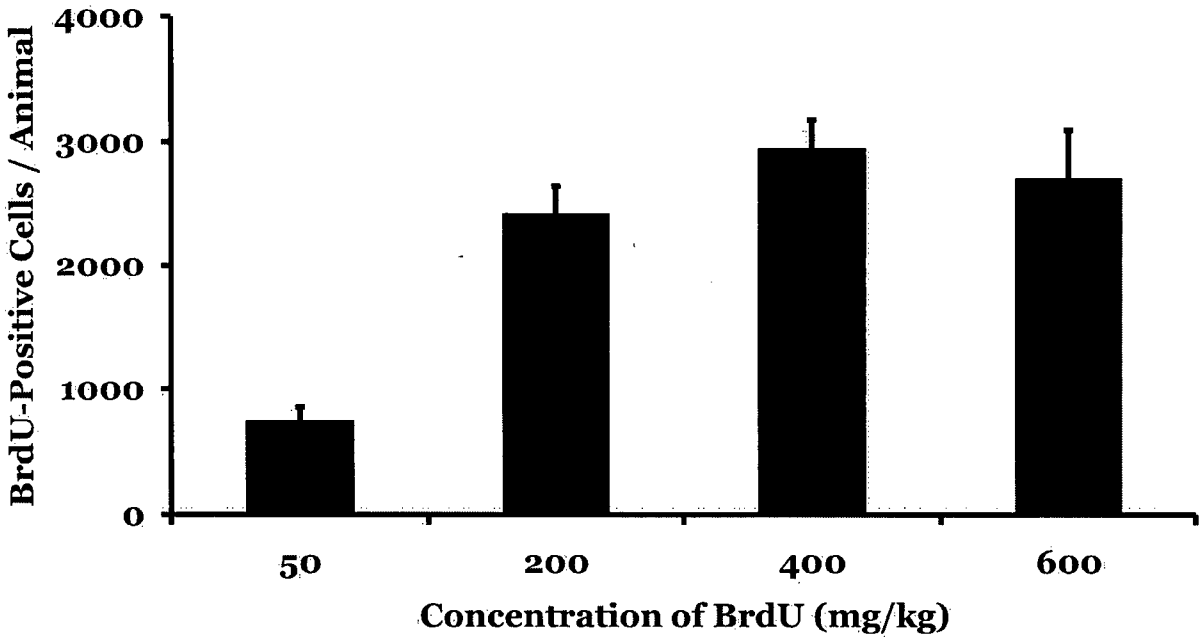

Figure 2. Total number of BrdU-positive cells in the subgranular zone of the dentate gyrus 24 hours following a single injection of BrdU at a concentration of either 50, 200, 400, or 600 mg/kg in voluntary exercise animals. Animals that have had free access to a running wheel show saturating levels of BrdU-positive cells when a dosage of 200 mg/kg of BrdU was employed.

	50 mg/kg	200 mg/kg	400 mg/kg	600 mg/kg	p
Con	480 (143)	1651 (323)	1296 (277)	1776 (96)	*< 0.05*
VE	730 (125)	2409 (237)	2928 (245)	2688 (402)	*< 0.05*
P	*NS*	*< 0.05*	*< 0.05*	*< 0.05*	

Table 1. Mean number of BrdU-positive cells in the subgranular zone of the dentate gyrus in voluntary exercise and control animals across a variety of dosages of BrdU. A consistent and significant increase in the number of BrdU-positive cells in voluntary exercise animals was observed at three saturating doses of BrdU (200, 400, and 600 mg/kg), but not at a sub-saturating dose (50 mg/kg). Standard error of the mean values are reported in parantheses.

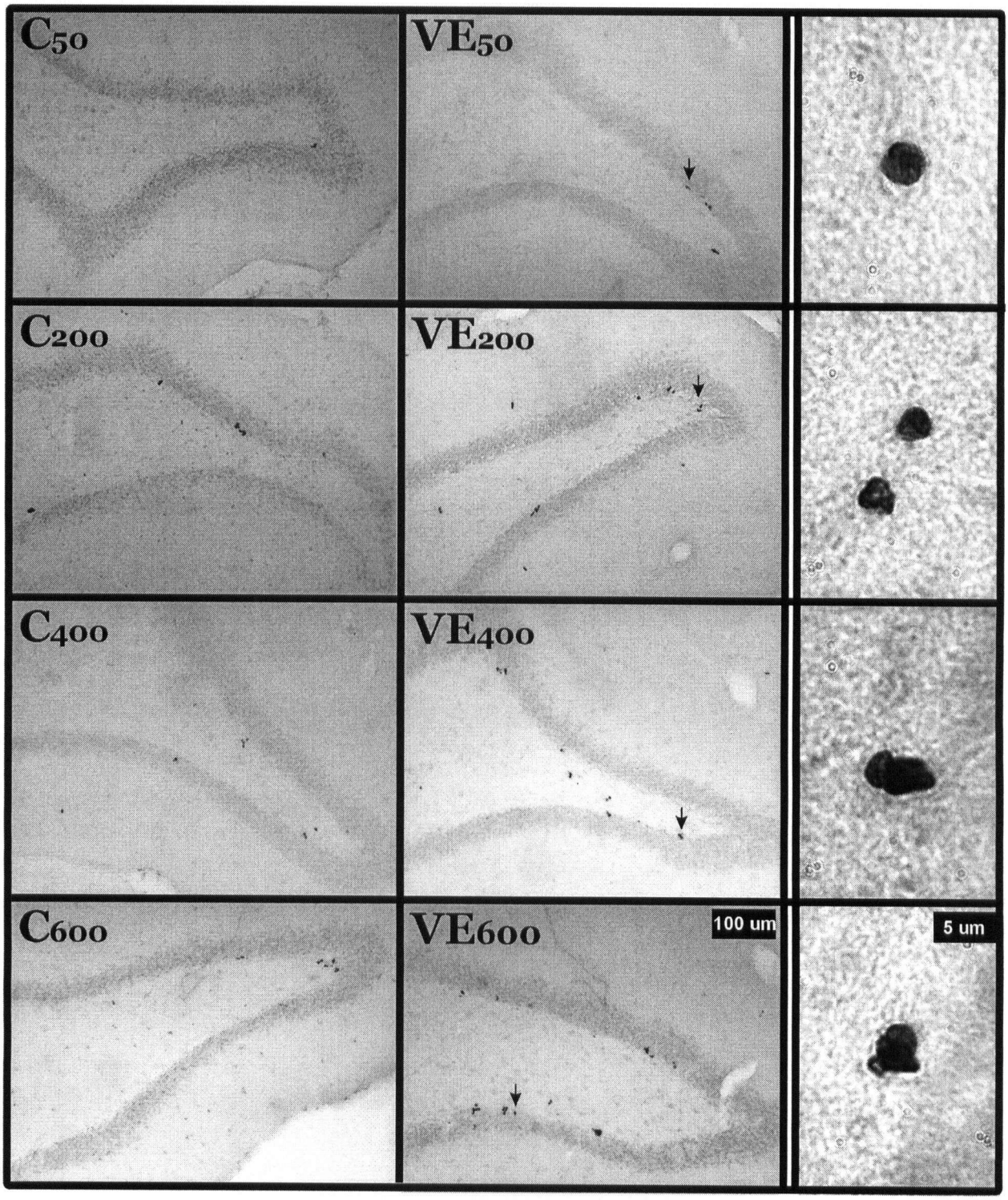

Figure 3. BrdU labeled cells in the granule cell layer and subgranular zone of the dentate gyrus and did not alter the appearance of individual cells, regardless of the dose of BrdU used. Representative pictures (10x magnification) of BrdU-labeled cells in the dentate gyrus is shown for all doses of BrdU in controls (C_{50}, C_{200}, C_{400}, and C_{600}; left column) and voluntary exercise animals (VE_{50}, VE_{200}, VE_{400}, and VE_{600}; middle column). Arrows indicate the cell(s) that is shown at 100x magnification in the far right column. The scale bars are 100 μm for the pictures in the left and middle columns, and 10 μm for pictures in the right column.

twice as many Ki67-positive cells in the subgranular zone of the DG (Voluntary Exercise, 6470 ±521; Control, 2620 ±431; $T_{(11)} = 6.23$, $p<0.05$). Thus, the intrinsic marker showed an increase with VE that was proportional to that which we observed using saturating doses of BrdU. Furthermore, the Ki67 immuno-labeled cells had a similar morphology to BrdU immuno-labeled cells (Fig. 4).

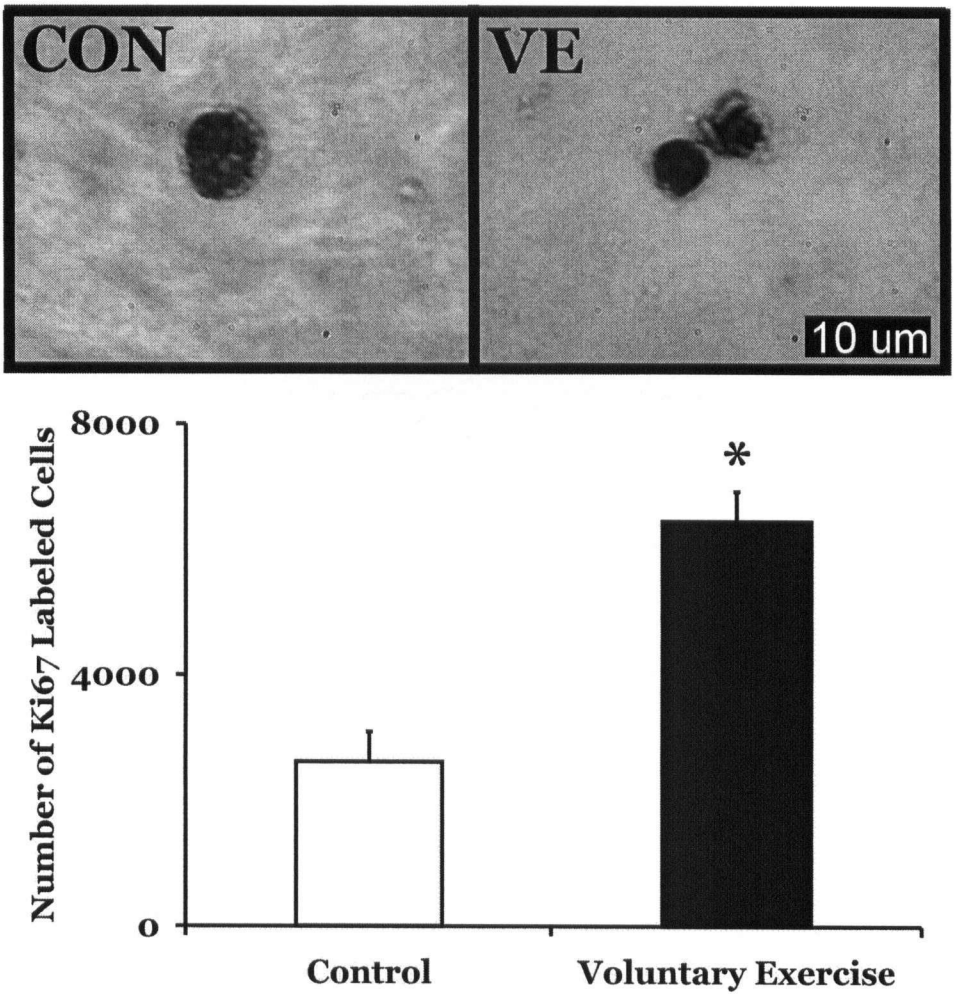

Figure 4. Voluntary exercise leads to an increase in the total number of Ki67-labeled cells in the dentate gyrus of adult male rats, and clearly labels cells in the dentate gyrus. Representative pictures, taken under 200x magnification, of Ki67-positive cells in the dentate gyrus of control (CON) and voluntary exercise (VE) animals. Scale bar is 10um. Quantification shows that exposure to a running wheel for 2 weeks more than doubles the number of Ki67-labeled cells in the subgranular zone of the dentate gyrus. Asterick denotes $p<0.05$.

To determine whether VE alters other aspects of cellular morphology in the adult mammalian brain, we also used a modified-Golgi Cox procedure (Gibb & Kolb, 1998). This procedure produces high contrast and thorough impregnation of neurons in the CNS, particularly hippocampal neurons, and provided us with excellent resolution for performing both Scholl analysis and high magnification spine counts. Analysis of individual granule cells revealed that VE significantly increased the length of granule cell dendrites ($T_{(58)} = 3.03$, $p<0.05$; Fig. 5). On average, dendrites in control animals were 156 $\pm$6 μm; while dendrites in VE animals were 181 $\pm$7 μm. This increase in length was due to changes in the distal dendrites, as Sholl analysis revealed that dendritic complexity was enhanced between 136 and 180 μm ($T_{(58)} = 2.70$, $p<0.05$) and 181 and 225 μm ($T_{(58)} = 2.52$, $p<0.05$) from the soma in VE animals. Furthermore, when spine counts were performed on the same tissue, it was revealed that spine density is increased from VE by approximately 29% ($T_{(58)} = 7.48$, $p<0.05$). We found, on average, 7.6 $\pm$0.13 spines per 10 μm in controls, and 9.8 $\pm$0.11 spines per 10 μm in VE animals. Thus, in addition to increases in the length and complexity of the dendrites of dentate granule neurons following VE, there is also a marked increase in the number of spines available for making synaptic contacts.

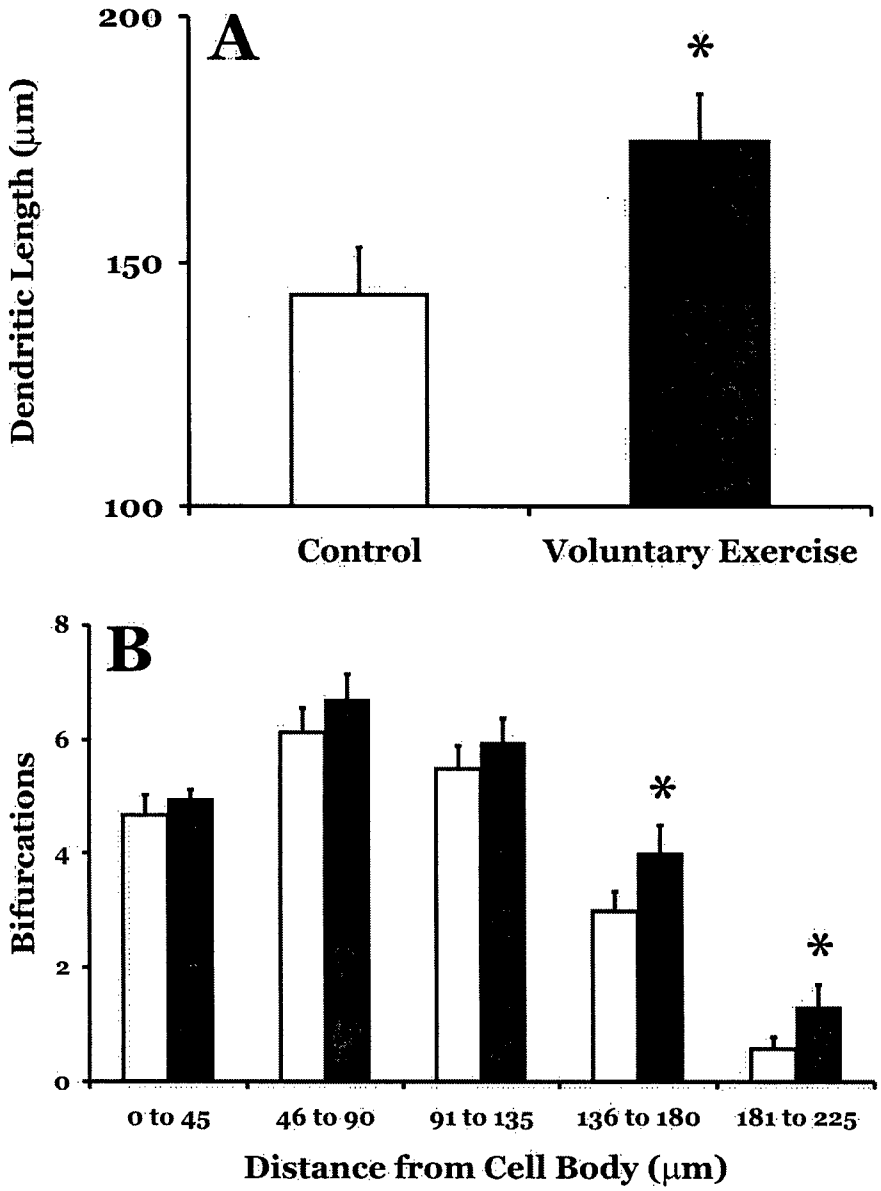

Figure 5. Voluntary exercise increases dendritic length and distal bifurcations in the dentate gyrus. The length of dendrites extending through the molecular layer of the dentate gyrus is significantly longer in animals that have been exposed to a running wheel (A). In addition, voluntary exercise significantly increases dendritic complexity in the distal regions of the molecular layer (B). Asterix denote $p<0.05$.

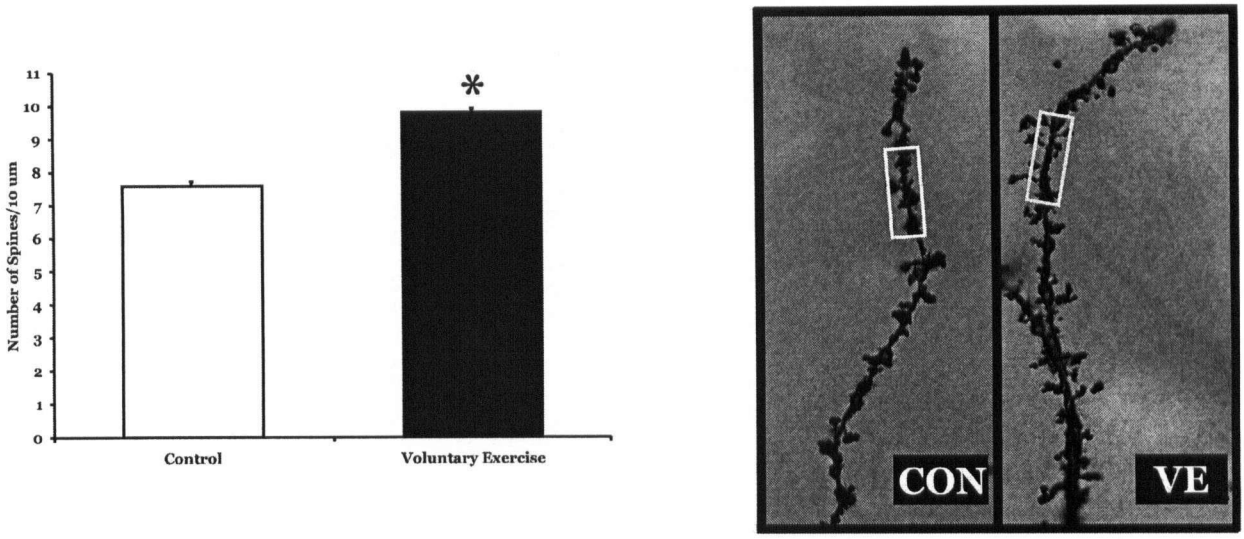

Figure 6. Voluntary exercise significantly increases spine density in the dentate gyrus of the hippocampus. Mean number of dendritic spines per 10 μm units (white boxes) on dendritic processes of dentate granule neurons. Voluntary exercise significantly enhances the number of dendritic spines in the dentate gyrus. Pictures shown are representative samples of dendrites of golgi-impregnated dentate granule neurons taken at 100x magnification, from a control (CON) and voluntary exercise (VE) animal. Asterik denotes $p<0.05$.

DISCUSSION

This paper demonstrates that structural plasticity is enhanced in the adult DG in response to voluntary exercise, and that there is an increase in the pool of new cells as well as a change in the dendritic morphology of existing cells.

Using an exogenous marker of cellular proliferation (BrdU) in the DG of the adult rat brain, we found that VE substantially increased the number of new cells generated. In an earlier study, we employed a dosage of BrdU (50 mg/kg) that, in light of new evidence, may be *non*-saturating, though we did administer the BrdU repeatedly across several days. Even with multiple injections, there would be dividing cells that are not labeled following each BrdU injection. Because exercise has been shown to induce angiogenesis (Kleim et al., 2002; Swain et al., 2003), increase both cerebral blood volume and blood flow (Swain et al., 2003; Yancey & Overton, 1993), and transiently increase the permeability of the BBB (Sharma et al., 1991), VE possesses the potential to increase the length of time and quantity of BrdU that reaches the brain. This in turn could influence the number of dividing cells that are labeled in a given population of proliferating cells. In the current study, we injected VE animals and controls with 50 mg/kg of BrdU and found that VE did not significantly affect the total number of BrdU-positive cells ($p=0.09$). The difference between this result and our previous work may be a function of the fact that we previously administered multiple injections to young, female C57/Bl6 mice. In this study, we show that relatively higher doses of BrdU (200, 400, and 600 mg/kg) label significantly more cells. Clearly, employment of sub-saturating doses of BrdU can lead to false conclusions, possibly because variability is increased when only a fraction of the total cell population is labeled. Thus, as noted by Cameron and McKay (2001), higher doses of BrdU are warranted to ensure that cells that are dividing at a given point in time are labeled.

By comparing the number of BrdU-positive cells using a saturating dose of BrdU in controls with the number of BrdU-positive cells using a saturating dose of BrdU in VE animals,

we showed a consistent and asymptotic increase in the number of labeled cells in the VE animals. This is a result that we would not expect if exercise altered BrdU access to, and/or uptake by, individual cells. This data strongly suggests that the observed increase in BrdU-positive cells in response to VE reflects an increase in cellular proliferation that is not caused by an enhancement of the metabolic processing and labeling of dividing cells.

Importantly, in the process of assessing how this effect responds across a variety of doses of BrdU, we determined that the minimal dose of BrdU required to label all dividing cells in adult, male Sprague-Dawley rats, was approximately 200 mg/kg in both control and VE animals. The main difference between the two groups was that the ceiling of the asymptote was increased substantially by VE. More importantly, this increase in BrdU-labeling did not instigate a significant increase in cell labeling outside of the subgranular zone of the DG. These findings corroborate and extend those from Cameron and McKay's (2001) recent report suggesting that a saturating dose of BrdU lies between 100 and 300 mg/kg and that high doses (> 400 mg/kg) do not obfuscate results.

In addition to the work done here with BrdU, we also show that the number of Ki67-labeled cells in the DG is increased in VE animals. Ki67 is a cell cycle protein that is expressed during all stages of the cell cycle except for Go and, perhaps, early G_1, that has been utilized as an endogenous marker of cellular proliferation (Scholzen & Gerdes, 2000). One of the advantages that this method has over the BrdU method, in regards to assessing cellular proliferation, is circumvention of possible confounds associated with vascular influences on the processing of an injected compound (as described above). Kee et al. (2002) have recently shown that the total number of Ki67-positive cells reflects cellular proliferation in a manner consistent with BrdU-labeling and that both methods yield quantitatively similar measures of ischemia and radiation induced changes in cellular proliferation. Ischemia and radiation lead to physiological changes that, like VE, may alter BrdU metabolism/uptake. We found significantly more Ki67

cells than BrdU-labeled cells in both controls and VE groups, consistent with findings from Kee et al. (2002). This is expected as Ki67 is expressed during much of the active cell cycle, whereas BrdU can only label cells that are in S-phase of the cell cycle for a limited time following injection. While a better understanding of the function of this protein in the cell will improve our ability to interpret this result, converging evidence supports the assumption that Ki67 reflects cell proliferation. The fact that we observed an increase in the number of Ki67-positive cells following VE, similar to that found using the BrdU method, provides further support that VE does increase cellular proliferation in the adult DG.

An important caveat to these findings is that, although the BrdU technique itself does not appear to be confounded by other physiological effects of VE, these other effects may in fact be critical to the increase in cellular proliferation. For example, the availability and permeability of the BBB to circulating growth factors, such as VEGF and IGF1, appears to be critical to the enhancement of neurogenesis in response to exercise (Aberg, Aberg, Hedbacker, Oscarsson, & Eriksson, 2000; Aberg et al., 2003; Fabel et al., 2003; Trejo, Carro, & Torres-Aleman, 2001).

In addition to increases in cellular proliferation, we report that VE alters the morphology of the dendrites of dentate granule cells. Using a modified Golgi-Cox technique (Gibb & Kolb, 1998), we showed that both the total length and complexity of dendrites in the DG are increased following VE. In addition, the density of spines on these dendrites was increased. Thus, for the first time, we report that VE enhances neuroplasticity in the DG via alterations to dendritic morphology. Even if there are no accompanying changes in channel number and composition, changes in dendritic morphology can drastically change the capacity of cells to process information (Mainen & Sejnowski, 1995, 1996). Interestingly, *in vivo* and *in vitro* recordings taken from the DG reveal that VE markedly increases LTP, a reflection of enhanced synaptic plasticity and the leading cellular model of learning and memory (Farmer et al., 2004; van Praag, Christie et al., 1999). In addition, animals that have been exposed to a running wheel show

improved performance on behavioural tasks of learning and memory, as do younger animals (Barnes and McNaughton 1985; van Praag, Christie, et al. 1999; Anderson et al., 2000). It has been suggested that this cognitive enhancement results from an increase in neurogenesis as we have shown that new cells become functional entities in the adult brain (van Praag et al., 2002). However, the present results suggest that there are likely changes to the composition of pre-existing cells that can alter the information processing power of the DG, and that this may also underlie the behavioral changes that have been observed. It is likely that both an increase in the production of new cells and an enhancement of the morphology of dendrites of dentate granule neurons, in addition to changes at the molecular level, underlie our early finding that voluntary exercise improves performance on hippocampal-dependent learning (Farmer et al., 2004; van Praag, Christie et al., 1999).

Previously, our laboratory has reported that VE increases levels of BDNF, and there is evidence that this increase in BDNF may be responsible for the observed increases in neurogenesis and LTP (Neeper, Gomez-Pinilla et al. 1995; Neeper, Gomez-Pinilla et al. 1996; Russo-Neustadt, Beard et al. 1999; van Praag, Christie et al. 1999; Berchtold, Kesslak et al. 2001; Farmer, Zhao et al. 2004). BDNF can alter dendritic morphology both *in vivo* and *in vitro* (Ang et al., 2003; Aoyagi et al., 1994; Barde, 1994; Berchtold et al., 2001; Farmer et al., 2004; McAllister et al., 1997; McAllister et al., 1995; Neeper et al., 1995; Neeper et al., 1996; Russo-Neustadt et al., 1999; Smith & Zigmond, 2003; Tolwani et al., 2002; Tyler & Pozzo-Miller, 2003; Tyler & Pozzo-Miller, 2001). Interestingly, Tolwani et al. (2002) have also shown that over-expression of BDNF in mice leads to an increase in the length and complexity of dendrites of dentate granule cells. Thus, it seems likely that there is a role for BDNF in the findings observed here. BDNF also affects the vasculature and this too could play a role in the enhanced structural and functional plasticity observed following VE (Donovan et al., 2000; Gustafsson, Lindvall, & Kokaia, 2003).

The present results may have important implications for the role of exercise in the recovery from neurotrauma and prevention of neurodegeneration. For example, Stummer et al. (1994) have shown that free access to a running wheel increases probability of survival following ischemia and attenuates damage in the dentate gyrus. In addition, VE between 14 and 20 days post-traumatic brain injury increased hippocampal BDNF and improved performance on a hippocampal-dependent learning task (Griesbach, Hovda, Molteni, Wu, & Gomez-Pinilla, 2004). Voluntary exercise has also been shown to decrease learned helplessness/behavioral depression (Greenwood et al., 2003), and a novel theory of depression suggests that symptoms are related to decreases in neurogenesis in the DG (Jacobs, Praag, & Gage, 2000). In a recent prospective study, exercise was also shown to reduce the risk of cognitive impairment, Alzheimer's disease, and dementia of any type (Laurin, Verreault, Lindsay, MacPherson, & Rockwood, 2001), which have all been associated with hippocampal atrophy (Gallagher, Bizon, Hoyt, Helm, & Lund, 2003; Pennanen et al., 2004). Thus, the benefits of exercise in recovery from neurotrauma and prevention of neurodegeneration may be mediated, at least in part, by enhanced plasticity in the dentate gyrus.

CONCLUSION

Voluntary exercise induces morphological changes to the DG of the adult mammalian brain via (1) increases in neurogenic activity, and (2) increases in the dendritic complexity and density of dendritic spines of granule neurons. Future studies should seek to determine the relationships between these processes that allow for VE to enhance synaptic transmission in the hippocampus and improve behavior and cognition associated with this region of the brain. The results of this study support the employment of exercise as a viable option for prevention and recovery from disorders or insults affecting the dentate gyrus, and suggest that significant morphological changes aside from neurogenesis may be important for these beneficial effects. Neuroprotection and/or facilitated recovery from insults to the adult hippocampus can be achieved by exercise, and these benefits may not need to wait for the generation and incorporation of new neurons.

Literature Cited

Aberg, M. A., Aberg, N. D., Hedbacker, H., Oscarsson, J., & Eriksson, P. S. (2000). Peripheral infusion of IGF-I selectively induces neurogenesis in the adult rat hippocampus. *J Neurosci, 20*(8), 2896-2903.

Aberg, M. A., Aberg, N. D., Palmer, T. D., Alborn, A. M., Carlsson-Skwirut, C., Bang, P., et al. (2003). IGF-I has a direct proliferative effect in adult hippocampal progenitor cells. *Mol Cell Neurosci, 24*(1), 23-40.

Anderson, B. J., Rapp, D. N., Baek, D. H., McCloskey, D. P., Coburn-Litvak, P. S., & Robinson, J. K. (2000). Exercise influences spatial learning in the radial arm maze. *Physiol Behav, 70*(5), 425-429.

Ang, E. T., Wong, P. T., Moochhala, S., & Ng, Y. K. (2003). Neuroprotection associated with running: is it a result of increased endogenous neurotrophic factors? *Neuroscience, 118*(2), 335-345.

Brown, J., Cooper-Kuhn, C. M., Kempermann, G., Van Praag, H., Winkler, J., Gage, F. H., et al. (2003). Enriched environment and physical activity stimulate hippocampal but not olfactory bulb neurogenesis. *Eur J Neurosci, 17*(10), 2042-2046.

Cameron, H. A., & McKay, R. D. (2001). Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. *J Comp Neurol, 435*(4), 406-417.

Donovan, M. J., Lin, M. I., Wiegn, P., Ringstedt, T., Kraemer, R., Hahn, R., et al. (2000). Brain derived neurotrophic factor is an endothelial cell survival factor required for intramyocardial vessel stabilization. *Development, 127*(21), 4531-4540.

Fabel, K., Tam, B., Kaufer, D., Baiker, A., Simmons, N., Kuo, C. J., et al. (2003). VEGF is necessary for exercise-induced adult hippocampal neurogenesis. *Eur J Neurosci, 18*(10), 2803-2812.

Faherty, C. J., Kerley, D., & Smeyne, R. J. (2003). A Golgi-Cox morphological analysis of neuronal changes induced by environmental enrichment. *Brain Res Dev Brain Res, 141*(1-2), 55-61.

Farmer, J., Zhao, X., van Praag, H., Wodtke, K., Gage, F. H., & Christie, B. R. (2004). Effects of voluntary exercise on synaptic plasticity and gene expression in the dentate gyrus of adult male Sprague-Dawley rats in vivo. *Neuroscience, 124*(1), 71-79.

Gallagher, M., Bizon, J. L., Hoyt, E. C., Helm, K. A., & Lund, P. K. (2003). Effects of aging on the hippocampal formation in a naturally occurring animal model of mild cognitive impairment. *Exp Gerontol, 38*(1-2), 71-77.

Gibb, R., & Kolb, B. (1998). A method for vibratome sectioning of Golgi-Cox stained whole rat brain. *J Neurosci Methods, 79*(1), 1-4.

Greenwood, B. N., Foley, T. E., Day, H. E., Campisi, J., Hammack, S. H., Campeau, S., et al. (2003). Freewheel running prevents learned helplessness/behavioral depression: role of dorsal raphe serotonergic neurons. *J Neurosci, 23*(7), 2889-2898.

Griesbach, G. S., Hovda, D. A., Molteni, R., Wu, A., & Gomez-Pinilla, F. (2004). Voluntary exercise following traumatic brain injury: brain-derived neurotrophic factor upregulation and recovery of function. *Neuroscience, 125*(1), 129-139.

Gustafsson, E., Lindvall, O., & Kokaia, Z. (2003). Intraventricular infusion of TrkB-Fc fusion protein promotes ischemia-induced neurogenesis in adult rat dentate gyrus. *Stroke, 34*(11), 2710-2715.

Holmes, M. M., Galea, L. A., Mistlberger, R. E., & Kempermann, G. (2004). Adult hippocampal neurogenesis and voluntary running activity: circadian and dose-dependent effects. *J Neurosci Res, 76*(2), 216-222.

Jacobs, B. L., Praag, H., & Gage, F. H. (2000). Adult brain neurogenesis and psychiatry: a novel theory of depression. *Mol Psychiatry, 5*(3), 262-269.

Kee, N., Sivalingam, S., Boonstra, R., & Wojtowicz, J. M. (2002). The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis. *J Neurosci Methods, 115*(1), 97-105.

Kempermann, G., Kuhn, H. G., & Gage, F. H. (1997). More hippocampal neurons in adult mice living in an enriched environment. *Nature, 386*(6624), 493-495.

Kitamura, T., Mishina, M., & Sugiyama, H. (2003). Enhancement of neurogenesis by running wheel exercises is suppressed in mice lacking NMDA receptor epsilon 1 subunit. *Neurosci Res, 47*(1), 55-63.

Kleim, J. A., Cooper, N. R., & VandenBerg, P. M. (2002). Exercise induces angiogenesis but does not alter movement representations within rat motor cortex. *Brain Res, 934*(1), 1-6.

Laurin, D., Verreault, R., Lindsay, J., MacPherson, K., & Rockwood, K. (2001). Physical activity and risk of cognitive impairment and dementia in elderly persons. *Arch Neurol, 58*(3), 498-504.

Mainen, Z. F., & Sejnowski, T. J. (1995). Reliability of spike timing in neocortical neurons. *Science, 268*(5216), 1503-1506.

Mainen, Z. F., & Sejnowski, T. J. (1996). Influence of dendritic structure on firing pattern in model neocortical neurons. *Nature, 382*(6589), 363-366.

McAllister, A. K., Katz, L. C., & Lo, D. C. (1997). Opposing roles for endogenous BDNF and NT-3 in regulating cortical dendritic growth. *Neuron, 18*(5), 767-778.

McAllister, A. K., Lo, D. C., & Katz, L. C. (1995). Neurotrophins regulate dendritic growth in developing visual cortex. *Neuron, 15*(4), 791-803.

McKittrick, C. R., Magarinos, A. M., Blanchard, D. C., Blanchard, R. J., McEwen, B. S., & Sakai, R. R. (2000). Chronic social stress reduces dendritic arbors in CA3 of hippocampus and decreases binding to serotonin transporter sites. *Synapse, 36*(2), 85-94.

Miller, M. W., & Nowakowski, R. S. (1988). Use of bromodeoxyuridine-immunohistochemistry to examine the proliferation, migration and time of origin of cells in the central nervous system. *Brain Res, 457*(1), 44-52.

Neeper, S. A., Gomez-Pinilla, F., Choi, J., & Cotman, C. (1995). Exercise and brain neurotrophins. *Nature, 373*(6510), 109.

Neeper, S. A., Gomez-Pinilla, F., Choi, J., & Cotman, C. W. (1996). Physical activity increases mRNA for brain-derived neurotrophic factor and nerve growth factor in rat brain. *Brain Res, 726*(1-2), 49-56.

Overstreet, L. S., Hentges, S. T., Bumaschny, V. F., de Souza, F. S., Smart, J. L., Santangelo, A. M., et al. (2004). A transgenic marker for newly born granule cells in dentate gyrus. *J Neurosci, 24*(13), 3251-3259.

Pennanen, C., Kivipelto, M., Tuomainen, S., Hartikainen, P., Hanninen, T., Laakso, M. P., et al. (2004). Hippocampus and entorhinal cortex in mild cognitive impairment and early AD. *Neurobiol Aging, 25*(3), 303-310.

Rhodes, J. S., van Praag, H., Jeffrey, S., Girard, I., Mitchell, G. S., Garland, T., Jr., et al. (2003). Exercise increases hippocampal neurogenesis to high levels but does not improve spatial learning in mice bred for increased voluntary wheel running. *Behav Neurosci, 117*(5), 1006-1016.

Russo-Neustadt, A., Beard, R. C., & Cotman, C. W. (1999). Exercise, antidepressant medications, and enhanced brain derived neurotrophic factor expression. *Neuropsychopharmacology, 21*(5), 679-682.

Schmidt-Hieber, C., Jonas, P., & Bischofberger, J. (2004). Enhanced synaptic plasticity in newly generated granule cells of the adult hippocampus. *Nature, 429*(6988), 184-187.

Scholzen, T., & Gerdes, J. (2000). The Ki-67 protein: from the known and the unknown. *J Cell Physiol, 182*(3), 311-322.

Sharma, H. S., Cervos-Navarro, J., & Dey, P. K. (1991). Increased blood-brain barrier permeability following acute short-term swimming exercise in conscious normotensive young rats. *Neurosci Res, 10*(3), 211-221.

Sholl, D. A. (1956). The measurable parameters of the cerebral cortex and their significance in its organization. *Prog Neurobiol*(2), 324-333.

Smith, A. D., & Zigmond, M. J. (2003). Can the brain be protected through exercise? Lessons from an animal model of parkinsonism. *Exp Neurol, 184*(1), 31-39.

Snyder, J. S., Kee, N., & Wojtowicz, J. M. (2001). Effects of adult neurogenesis on synaptic plasticity in the rat dentate gyrus. *J Neurophysiol, 85*(6), 2423-2431.

Steiner, B., Kronenberg, G., Jessberger, S., Brandt, M. D., Reuter, K., & Kempermann, G. (2004). Differential regulation of gliogenesis in the context of adult hippocampal neurogenesis in mice. *Glia, 46*(1), 41-52.

Swain, R. A., Harris, A. B., Wiener, E. C., Dutka, M. V., Morris, H. D., Theien, B. E., et al. (2003). Prolonged exercise induces angiogenesis and increases cerebral blood volume in primary motor cortex of the rat. *Neuroscience, 117*(4), 1037-1046.

Takahashi, T., Nowakowski, R. S., & Caviness, V. S., Jr. (1992). BUdR as an S-phase marker for quantitative studies of cytokinetic behaviour in the murine cerebral ventricular zone. *J Neurocytol, 21*(3), 185-197.

Tolwani, R. J., Buckmaster, P. S., Varma, S., Cosgaya, J. M., Wu, Y., Suri, C., et al. (2002). BDNF overexpression increases dendrite complexity in hippocampal dentate gyrus. *Neuroscience, 114*(3), 795-805.

Trejo, J. L., Carro, E., & Torres-Aleman, I. (2001). Circulating insulin-like growth factor I mediates exercise-induced increases in the number of new neurons in the adult hippocampus. *J Neurosci, 21*(5), 1628-1634.

Turner, C. A., & Lewis, M. H. (2003). Environmental enrichment: effects on stereotyped behavior and neurotrophin levels. *Physiol Behav, 80*(2-3), 259-266.

Tyler, W. J., & Pozzo-Miller, L. (2003). Miniature synaptic transmission and BDNF modulate dendritic spine growth and form in rat CA1 neurones. *J Physiol, 553*(Pt 2), 497-509.

Tyler, W. J., & Pozzo-Miller, L. D. (2001). BDNF enhances quantal neurotransmitter release and increases the number of docked vesicles at the active zones of hippocampal excitatory synapses. *J Neurosci, 21*(12), 4249-4258.

van Praag, H., Christie, B. R., Sejnowski, T. J., & Gage, F. H. (1999). Running enhances neurogenesis, learning, and long-term potentiation in mice. *Proc Natl Acad Sci U S A, 96*(23), 13427-13431.

van Praag, H., Kempermann, G., & Gage, F. H. (1999). Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. *Nat Neurosci, 2*(3), 266-270.

van Praag, H., Schinder, A. F., Christie, B. R., Toni, N., Palmer, T. D., & Gage, F. H. (2002). Functional neurogenesis in the adult hippocampus. *Nature, 415*(6875), 1030-1034.

Yancey, S. L., & Overton, J. M. (1993). Cardiovascular responses to voluntary and treadmill exercise in rats. *J Appl Physiol, 75*(3), 1334-1340.