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Endogenous Cannabinoid Signaling Is Required for Voluntary Exercise-induced Enhancement of Progenitor Cell Proliferation in the Hippocampus

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Abstract

Voluntary exercise and endogenous cannabinoid activity have independently been shown to regulate hippocampal plasticity. The aim of the current study was to determine whether the endocannabinoid system is regulated by voluntary exercise and if these changes contribute to exercise-induced enhancement of cell proliferation. In Experiment 1, eight days of free access to a running wheel increased the agonist binding site density of the cannabinoid CB_1 receptor; CB_1 receptor-mediated GTPγS binding; and the tissue content of the endocannabinoid anandamide in the hippocampus but not in the prefrontal cortex. In Experiment 2, the CB_1 receptor antagonist AM251 (1 mg/kg) was administered daily to animals given free access to a running wheel for 8 days, after which cell proliferation in the hippocampus was examined through immunohistochemical analysis of the cell cycle protein Ki-67. Voluntary exercise increased proliferation of progenitor cells, as evidenced by the increase in the number of Ki-67 positive cells in the granule cell layer of the dentate gyrus in the hippocampus. However, this effect was abrogated by concurrent treatment with AM251, indicating that the increase in endocannabinoid signaling in the hippocampus is required for the exercise-induced increase in cell proliferation. These data demonstrate that the endocannabinoid system in the hippocampus is sensitive to environmental change and suggest that it is a mediator of experience-induced plasticity.

Keywords

cell proliferation; antidepressant; running; 2-AG; FAAH; neuroprotection

Introduction

The development of new neurons, or neurogenesis, occurs when neural progenitor cells in the subgranular zone of the dentate gyrus (as well as in the olfactory bulb) undergo mitosis to produce a population of daughter cells, which primarily assume a neuronal phenotype, although some do become glia (Christie and Cameron, 2006; Zhao et al., 2008). As the new hippocampal neurons mature, to become functional they must extend axons and dendrites

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into the established cytoarchitecture of the existing neuronal networks, and also develop appropriate bioelectrical properties and connections for dentate granule cells (Lledo et al., 2006; Schmidt-Hieber et al., 2004; van Praag et al., 2002).

Cell proliferation and neurogenesis in the hippocampus are sensitive to regulation by a number of environmental factors, including stress, enrichment and exercise (Mirescu and Gould, 2006; Olson et al., 2006). Voluntary exercise (VEx) is a particularly robust way to enhance progenitor cell proliferation and neurogenesis, possibly because it also increases a myriad of physiological responses that include neurotrophin expression, dendritic length and complexity, spine density, angiogenesis and cerebral blood flow, within the dentate gyrus (Eadie et al., 2005; Farmer et al., 2004; Neeper et al., 1996; Pereira et al., 2007; Redila and Christie, 2006; Stranahan et al., 2007; van Praag et al., 1999). As a result of these changes, animals that exercise show enhanced long-term potentiation, as well as observable improvements in performance on cognitive tasks (van Praag et al., 1999; Vaynman et al., 2004, 2007).

Converging lines of evidence also support a role of the endocannabinoid system in neuroplastic phenomena, particularly within the hippocampus (Aguado et al., 2005, 2006; Hashimotodani et al., 2007; Zhu, 2006). The endocannabinoid system is a neuromodulatory system composed of two receptors (CB_1 and CB_2) and the arachidonate-derived endogenous ligands, *N*-arachidonylethanolamide (anandamide; AEA) and 2-arachidonoylglycerol (2-AG) (Hillard, 2000; Howlett et al., 2004). Both *in vitro* and *in vivo* studies have revealed that neural progenitor cells express both CB_1 and CB_2 receptors and synthesize AEA and 2-AG (Aguado et al., 2005, 2006; Jiang et al., 2005; Molina-Holgado et al., 2007; Palazuelos et al., 2006). Genetic deletion of the CB_1 receptor suppresses progenitor cell proliferation (Aguado et al., 2005, 2006; Jin et al., 2004; Kim et al., 2006), while genetic deletion of the enzyme responsible for AEA hydrolysis (fatty acid amide hydrolase; FAAH) results in a profound increase in cell proliferation within the dentate gyrus (Aguado et al., 2005, 2006). In addition to these effects on progenitor cell proliferation, the endocannabinoid system interacts with several neurotrophic systems (Aso et al., 2008; Khaspekov et al., 2004; Williams et al., 2003), making this system an ideal candidate for mediating the effects of VEx on hippocampal cytogenesis. Consistently, in addition to the ability of VEx to increase circulating growth factors (Schwarz et al., 1996; Schobersberger et al., 2000), AEA increases in the circulation following a sustained period of physical exercise in humans (Sparling et al., 2003), demonstrating that exercise activates the endocannabinoid system. A recent report has also suggested that endocannabinoid signaling is involved in the rewarding properties of running (Keeney et al., 2008), indicating that functional associations may exist between VEx and the endocannabinoid system. Therefore, the current study sought to determine if VEx modulates hippocampal endocannabinoid signaling, and the extent to which these changes contribute to alterations in neuroplasticity following exercise.

Methods

Subjects

Seventy day old male Sprague-Dawley rats (300 g; Charles River Laboratories, Montreal, Canada) were housed in groups of three in triple mesh wire caging for a ten day acclimation period following arrival. After acclimation, half of the rats were randomly assigned to either standard caging (containing enrichment in the form of PVC tubing) or caging that contained a running wheel connected to a PC computer (Mini-Mitter Systems Inc., WA, USA). Animals in the VEx condition were housed in cages with running wheels for eight days while control animals were housed individually in equivalent caging without a running wheel for the same period of time. Colony rooms were maintained at 21 °C, and on a 12 h light/dark cycle, with lights on at 0900 h. All rats were given *ad libitum* access to Purina Rat

Chow and tap water. All protocols were approved by the Canadian Council for Animal Care and the Animal Care Committee of the University of British Columbia.

Experiment 1: The Effects of VEx on the Endocannabinoid System in the Hippocampus and Prefrontal Cortex—Animals were housed and maintained as described above. VEx animals and sedentary control animals were sacrificed between 0900-1100 h on the morning following eight days of access to running wheels. A diagram of the treatment conditions can be seen in Fig. 1. The hippocampus and prefrontal cortex (consisting of medial prefrontal cortex and anterior cingulate cortex) were sectioned out as previously described (Hill et al., 2006a), frozen in liquid nitrogen within 5 min of decapitation and stored at -80 °C until analysis. Two cohorts of tissue were collected. One cohort of tissue (n = 7) was used for lipid extraction to determine endocannabinoid ligand content. The other cohort of tissue (n = 5) was used to create membrane fractions for subsequent determination of CB_1 receptor binding, CB_1 receptor-mediated GTPγS binding and FAAH activity.

Membrane Preparation

Dissected brain sections were homogenized in 10 volumes of 0.32 M sucrose containing 3 mM HEPES (pH 7.5) and 1 mM EDTA. The homogenates were centrifuged at 18,000 × *g* for 20 min after which the supernatant was rapidly decanted. The remaining pellet, which is the membrane fraction, was resuspended in 1-2 ml TME buffer (50 mM Tris HCl, pH 7.4; 1 mM EDTA and 3 mM $MgCl_2$). Protein concentrations were determined by the Bradford method (Bio-Rad, Hercules, CA, USA).

CB_1 Receptor Binding Assay

CB_1 receptor binding assays were performed using a Multiscreen Filtration System with Durapore 1.2-μM filters (Millipore, Bedford, MA) as described previously (Hillard et al., 1995a). Incubations (total volume = 0.2 mL) were carried out using TME buffer containing 1 mg/mL bovine serum albumin (TME/BSA). Membranes (10 μg protein per incubate) were added to the wells containing 0.1, 0.25, 0.5, 1.0, 1.5 or 2.5 nM ^{3}H-CP 55,940, a cannabinoid CB_1 receptor agonist. Ten μM Δ^9-tetrahydrocannabinol was used to determine non-specific binding. K_D and Bmax values were determined by nonlinear curve fitting to the single site binding equation using GraphPad Prism (San Diego, CA, USA).

CB_1 Receptor-mediated GTPγS Binding Assay

The assay for [^{35}S]GTPγS binding was performed as previously described by Kearn et al. (1999). Briefly, membranes (final concentration, 5 μg of protein per incubation mixture) were added to TME buffer containing 0.1% fatty acid-free bovine serum albumin, 10 μmol/L GDP, and 150 mmol/L NaCl. [^{35}S]GTPγS (final concentration, 0.65 nmol/L) was added, and the incubation was continued for 30 min at 37°C using the Multiscreen Filtration System with Durapore filters (pore size, 1.2 μm; Millipore, Bedford, MA, USA). Non-specific binding was determined in the presence of 10 μmol/L Gpp(NH)p and accounted for <15% of the total binding. Bound [^{35}S]GTPγS was separated from free [^{35}S]GTPγS by filtration followed by washing the filters four times with cold TME buffer containing NaCl and GDP. The cannabinoid CB_1 receptor agonist WIN 55,212 was added in 1 μL of dimethyl sulfoxide at concentrations of 0, 0.1, 0.3, 0.6, 1, 2, 3, 6, 10, 20 and 30 μmol/L. In each experiment, the agonist-dependent [^{35}S]GTPγS binding was divided by agonist-independent binding and multiplied by 100 to convert to a percentage. The EC_{50} values and maximal agonist-induced increase in binding (E_{max}) of [^{35}S]GTPγS were determined by fitting the data to a sigmoidal concentration-response curve using nonlinear regression (Prism; GraphPad, San Diego, CA, USA).

Fatty Acid Amide Hydrolase Activity Assay

FAAH activity was measured as the conversion of AEA to arachidonic acid and ethanolamine by membrane preparations (Hillard et al., 1995b). AEA labeled with [^{3}H] in the ethanolamine portion of the molecule ([^{3}H]AEA; Omeir et al., 1995) was the radiolabeled substrate. Membranes were incubated in a final volume of 0.5 ml of TME buffer (50 mM Tris-HCl, 3.0 mM $MgCl_2$, and 1.0 mM EDTA, pH 7.4) containing 1.0 mg/ml fatty acid-free bovine serum albumin and 0.2 nM [^{3}H]AEA. Isotherms were constructed using eight concentrations of AEA at concentrations between 10 nM and 10 μM. Incubations were carried out at 37°C and were stopped with the addition of 2 ml of chloroform/methanol (1:2). After standing at ambient temperature for 30 min, 0.67 ml of chloroform and 0.6 ml of water were added. Aqueous and organic phases were separated by centrifugation at 1,000 rpm for 10 min. The amount of [^{3}H] in 1 ml each of the aqueous and organic phases was determined by liquid scintillation counting and the conversion of [^{3}H]AEA to [^{3}H]ethanolamine was calculated. The K_I and Vmax values for this conversion were determined by fitting the data to a single site competition equation using Prism. The r^2 value for the goodness of fit of the data to the single site, hyperbolic equation was always greater than 0.9 and typically closer to 0.98.

Endocannabinoid Extraction and Analysis

For analysis of endocannabinoid content, brain regions were subjected to a lipid extraction process as described previously (Patel et al., 2003). Briefly, tissue samples were weighed and placed into borosilicate glass culture tubes containing 2 ml of acetonitrile with 84 pmol of [$^2H_8$]anandamide and 186 pmol of [$^2H_8$]2-AG for extraction. Tissue was homogenized with a glass rod and sonicated for 30 min. Samples were incubated overnight at -20°C to precipitate proteins then centrifuged at 1,500 × *g* to remove particulates. The supernatants were removed to a new glass tube and evaporated to dryness under N_2 gas. The samples were resuspended in 300 μl of methanol to recapture any lipids adhering to the glass tube, and dried again under N_2 gas. Finally, lipid extracts were suspended in 20 μl of methanol, and stored at −80°C until analysis. The contents of the two primary endocannabinoids AEA and 2-AG within lipid extracts in methanol from brain tissue and plasma were determined using isotope-dilution liquid chromatography–mass spectrometry as described previously (Patel et al., 2005).

Experiment 2: The Role of the Endocannabinoid System in Exercise-induced Increase in Cell Proliferation in the Dentate Gyrus—Subjects were acclimated and assigned to groups as described above. Once in their respective housing conditions, half of the VEx and sedentary control received daily injections of the cannabinoid CB_1 receptor antagonist AM251 (1 mg/kg; Tocris Biosciences, Ellisville, MO, USA), while the other half received daily injections of vehicle (1:1:8 solution of DMSO: Tween 80: 0.9% saline). Injections began the morning following the initial transfer to the new cages and were performed using 26 gauge 1/2″ needles at a volume of 1 ml/kg. To minimize any disruption in activity levels, daily injections were given at the onset of the light cycle (between 0900-1100h), when the animals were the least active. A diagram of the treatment conditions can be seen in Fig. 1.

Following the morning of the eighth consecutive day of VEx, all subjects were overdosed with sodium pentobarbital (120 mg/kg) and perfused transcardially 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 obtained throughout the extent of the hippocampus with a Leica VT1000 vibratome.

Ki-67 Immunohistochemistry and Volumetric Analysis of the Dentate Gyrus

For Ki-67 immunohistochemistry, the tissue was rinsed in 0.1 M TBS, followed by 0.3% H_2O_2 and then transferred to the primary antibody solution containing 0.5% Triton X, 1% normal horse serum and 1:1000 rabbit anti-Ki-67 (Vector, Burlington ON, Canada) in 0.1 M TBS. Tissue was incubated in the primary solution for 20 h at room temperature (approximately 21 °C) and then rinsed in 0.1 M TBS. Tissue was then incubated in Biotinylated goat anti-rabbit IgG (Vector, Burlington ON, Canada) diluted 1:1000 in 0.1 M TBS for 1 h at room temperature and was then washed in 0.1 M TBS. An avidin-biotin peroxidase was applied for 60 min (ABC elite kit; Vector, Burlington ON, Canada). Labeling was visualized by incubating tissue in 5 mg/ml 3,3′-diaminobenzidine (DAB; Sigma-Aldrich, Oakville ON, Canada). Finally, the tissue was mounted on glass slides.

We utilized a modified stereological approach to determine the expression of Ki-67-labeled cells across the rostral-caudal extent of the hippocampus. Ki-67-labeled cells were counted in every 10th section (400 μm apart) throughout the neurogenic region of the granule cell layer (which is the subgranular zone and is defined as zero to two cell bodies from the inner edge of the molecular layer) and the hilus to obtain an estimate of the total number of labeled cells in each region. Counting was performed using a 100× oil immersion objective and a Nikon E600 light microscope. Corresponding area measurements were made of the dentate gyrus (granule cell layer and hilus calculated separately) using the software program Image J. Volume estimations of the dentate gyrus were then calculated using Cavalieri's principle (Gundersen and Jensen, 1987) by multiplying the aggregated areas by the distance between sections (400 μm). Total cell counts were calculated by multiplying the number of Ki-67-labeled cells per animal by 10. Densities of Ki-67-labeled cells per cubic millimeter in each region (granule cell layer or hilus) were also calculated by dividing the total number of Ki-67-labeled cells by the volume of the region (granule cell layer or hilus).

Statistical Analysis

For Experiment 1, the effects of VEx on the endocannabinoid system were analyzed by comparing VEx and sedentary control animals using an independent t-test. For Experiment 2, a univariate analysis of variance (ANOVA) was used with exercise and AM251 as fixed factors. Post hoc analysis was performed using a Tukey's test. Significance was established against an alpha level equal to 0.05.

Results

Exercise Increases Endocannabinoid Signaling in the Hippocampus

Animals with free access to a running wheel for eight days exhibited a significant increase in the maximal binding (B_{max}) of [^{3}H]CP55940 to the CB_1 receptor in the hippocampus [$t(8) = 5.29$, $p < 0.005$; Fig. 2 and Table 1]. This increase in the B_{max} of the CB_1 receptor was accompanied by a significant reduction in the affinity (K_d) of [^{3}H] CP55940 for the CB_1 receptor [$t(8) = 2.84$, $p < 0.05$; Fig. 2 and Table 1]. There was no effect of VEx on the B_{max} [$t(8) = 0.48$, $p > 0.05$; Table 1] or the K_d [$t(8) = 0.43$, $p > 0.05$; Table 1] of [^{3}H]CP55940 to bind to the CB_1 receptor in the prefrontal cortex.

VEx also increased CB_1 receptor mediated GTPγS binding [$t(8) = 3.63$, $p < 0.02$; Fig. 2 and Table 1]. The EC50 for the CB_1 receptor agonist, WIN55,212-2 was dramatically reduced in animals allowed VEx. VEx evoked a trend toward an increase in the maximal stimulation of GTPγS binding (E_{max}) elicited by the CB_1 receptor agonist WIN-55212,2 in the hippocampus [$t(8) = 1.94$, $p = 0.10$; Fig. 2 and Table 1].

The tissue content of AEA was significantly increased in the hippocampus following VEx [t (12) = 2.93, $p < 0.02$; Fig. 2]. This change was not associated with a decrease in the hydrolysis of AEA by FAAH because VEx had no effect on the maximal hydrolytic activity (V_{max}) of FAAH [t (8) = 0.55, $p > 0.05$; Table 2] or the binding affinity (K_m) of AEA for FAAH [t (8) = 0.66, $p > 0.05$; Table 2]. There was also a trend toward an increase in 2-AG content within the hippocampus [t (12) = 1.91, p = 0.08; Fig. 2]. Neither AEA [t (12) = 0.31, $p > 0.05$; Fig. 2] nor 2-AG [t (12) = 0.90, $p > 0.05$; Fig. 2] content was altered in samples from prefrontal cortex.

Exercise-induced Increase of Cell Proliferation in the Denate Gyrus requires Cannabinoid CB_1 Receptor Activity

Since VEx increases endocannabinoid signaling in the hippocampus and both endocannabinoid and physical activity can promote cell proliferation (Aguado et al., 2005, 2006; Eadie et al., 2005; Pereira et al., 2007), we examined if the exercise-induced increase in hippocampal endocannabinoid signaling contributes to enhanced cell proliferation in the dentate gyrus following VEx. Using the expression of an endogenous cell cycle protein, Ki-67, as a marker of neural progenitor cell proliferation, we found that there was a significant interaction between voluntary exercise and administration of AM251 on the total number of Ki-67 positive (Ki-67+) cells in the granule cell layer of the dentate gyrus [F (1, 19) = 7.40, $p < 0.02$; Fig. 3]. Post hoc analysis revealed that this interaction was due to an increase in Ki-67+ cells in the granule cell layer of animals that had engaged in VEx ($p < 0.04$); animals administered AM251, alone or in conjunction with VEx, did not exhibit any significant changes in Ki-67 expression in the dentate gyrus relative to vehicle treated controls ($p > 0.05$). This increased expression of Ki-67+ cells following VEx was not an artifact of structural changes within the dentate gyrus as there was neither a significant interaction [F (1, 19) = 0.25, $p > 0.05$; Table 3] nor main effect of either VEx [F (1, 19) = 0.35, $p > 0.05$] or AM251 administration [F (1, 19) = 1.90, $p > 0.05$] on the volume of the granule cell layer of the dentate gyrus.

When the expression of Ki-67+ cells was examined as a density measurement of the number of Ki-67+cells/mm^3 of the granule cell layer, an interaction was found between VEx and AM251 administration [F (1, 19) = 6.90, $p < 0.02$; data not shown]. Post hoc analyses revealed that VEx increased the density of Ki-67+ cells within the dentate gyrus ($p < 0.04$), which was abolished by administration of AM251. The effect of VEx and CB_1 receptor antagonism on cell proliferation was specific to the granule cell layer of the dentate gyrus as there was no significant interaction between exercise and AM251 [F (1, 19) = 1.39, $p > 0.05$; Fig. 3] nor main effects of either exercise [F (1, 19) = 1.14, $p > 0.05$] or AM251 administration [F (1, 19) = 0.00, $p > 0.05$] on the total number of Ki-67+ cells in the hilus of the dentate gyrus. Similarly, there was no effect of exercise and AM251 administration on the volume of the hilus of the dentate gyrus [F (1, 19) = 3.23, $p > 0.05$; Table 3] or the density of Ki-67+ cells within the hilus [F (1, 19) = 0.40, $p > 0.05$; data not shown]. Representative photomicrographs of the expression of Ki-67+ proliferating cells in the dentate gyrus following VEx, AM251 administration, or both treatments combined can be seen in Fig. 3.

It is important to note that there was no effect of drug treatment on the total distance run during the eight day running period [t (10) = 1.41, $p > 0.05$; data not shown] indicating that the reduction of proliferation following AM251 administration was not due to a reduction in VEx.

Discussion

These data demonstrate that VEx significantly increases endocannabinoid signaling within the hippocampus, and endocannabinoid signaling is required for VEx to increase proliferation of progenitor cells within the dentate gyrus. It is our hypothesis that these events are linked such that the effects of VEx on hippocampal endocannabinoid signaling drive the increase in progenitor cell proliferation. While the fate of these cells was not characterized in the present study, increased CB_1 receptor signaling has been found to promote both gliogenesis (Aguado et al., 2006) and neurogenesis (Jiang et al., 2005). However, an abundance of research has demonstrated that voluntary exercise promotes neurogenesis, with no changes in gliogenesis (Fabel et al. 2003; Olson et al., 2006; Pereira et al., 2007; van Praag et al., 1999), suggesting that the increase in cell proliferation documented in this study (and others) results in an increase in the production of new neurons. Consistent with this suggestion, it has been hypothesized that the mechanism by which VEx increases neurogenesis is through an up-regulation of cell proliferation in the dentate gyrus, thus producing a larger population of progenitor cells which can subsequently mature into neurons (Olson et al., 2006).

Rats with free access to a running wheel for eight days exhibited a significant increase in the agonist binding site density of the cannabinoid CB_1 receptor as revealed by the significant increase in the B_{max} of the CB_1 receptor; a significant increase in CB_1 receptor agonist potency to induce GTPγS binding as revealed by the significant reduction in the EC50 of WIN 55,212-2 to stimulate CB_1 receptor mediate GTPγS binding; and a significant increase in the hippocampal total tissue concentration of the endocannabinoid AEA. None of these effects were observed in the prefrontal cortex, indicating some degree of specificity to this phenomenon; however, it remains to be determined why these effects occurred within the hippocampus but not the prefrontal cortex. VEx increased the number of Ki-67+ cells in the granule cell layer of the dentate gyrus of the hippocampus, consistent with previous reports (Eadie et al., 2005; Fabel et al., 2003; Pereira et al., 2007; van Praag et al., 1999). However, the VEx-induced increase in cell proliferation did not occur in rats receiving daily administration of the cannabinoid CB_1 receptor antagonist, AM251 (1 mg/kg) throughout the eight day exercise period. Taken together, these data suggest a role for increased endocannabinoid signaling within the hippocampus by VEx in increasing mitotic activity within the granule cell layer of the dentate gyrus. It should be noted that the CB_2 receptor has also been identified to contribute to cell proliferation in the dentate gyrus (Palazuelos et al., 2006). While the current data do argue for an integral role of endocannabinoid signaling via the CB_1 receptor in the ability of exercise to augment cell proliferation in the hippocampus, it is also possible that endocannabinoid signaling via the CB_2 receptor may contribute to this phenomenon; however, further research is required to examine this question.

We recently reported that administration of the endocannabinoid uptake inhibitor/FAAH inhibitor, AM404, prevented stress-induced suppression of cell proliferation in the dentate gyrus (Hill et al., 2006b). Given that acute exposure to stress reduces AEA content within the hippocampus (Gorzalka et al., 2008), these data suggest that reductions in AEA/CB_1 receptor signaling contribute to the potential of stress to suppress cell proliferation. In light of the current study, these data, collectively, indicate that the endocannabinoid system, and particularly AEA/CB_1 receptor signaling, is an important mediator of experience-induced plasticity within the hippocampus, being both sensitive to environmental stimuli and a potent regulator of neuroplastic processes.

The mechanism(s) by which VEx increases endocannabinoid signaling remain to be determined. It is possible that the changes in receptor binding may be a result of changes in

ligand availability. Previous studies have indicated that the regulation of the cannabinoid CB_1 receptor and its endogenous ligands are not coupled in the typical negative regulation relationship, in which the ligand down-regulates its own receptor (Hill et al., 2005). In fact, direct infusion of 2-AG into the brain up-regulates CB_1 receptor mRNA transcription (Kola et al., 2005) and 2-AG stimulates membrane expression of CB_1 receptors in striatal tissue slices (Maccarrone et al., 2008), indicating that increased endocannabinoid signaling can promote both the genetic expression and surface recycling of the CB_1 receptor. Therefore, the VEx-induced increase of endocannabinoid content in the hippocampus observed in the current study could precede, and drive, the increase in the CB_1 receptor pool.

The increase in AEA content within the hippocampus, but not the prefrontal cortex, observed here was not due to a reduction in FAAH activity. Accordingly, it is not likely that the mechanism of action of this phenomenon involves decreased catabolism but rather is due to an enhancement in biosynthesis. Currently, there are three biochemical pathways that have been defined through which AEA synthesis can occur (Liu et al., 2006; Simon and Cravatt, 2006; Sun et al., 2004) and it is not known which of these is the predominant pathway in determining neuronal AEA synthesis, making it difficult to ascertain the enzymatic cascade responsible for mediating the increase in AEA following VEx. However, the synthesis of AEA is tightly coupled to calcium signaling and neuronal activation and is positively regulated by excitatory neurotransmission in the hippocampus (Jung et al., 2005; Marsicano et al., 2003; Ohno-Shosaku et al., 2002). Any type of movement, particularly running, would be expected to enhance network activity (i.e. theta activity) in the hippocampus (e.g., Keleman et al., 2005; Vanderwolf, 1969) and this may further enhance endocannabinoid synthesis and transmission. Alternately, cAMP-protein kinase signaling has been found to promote AEA synthesis in neuronal cultures and slice preparations (Azad et al., 2004; Cadas et al., 1996; Malcher-Lopes et al., 2006; Vellani et al., 2008) and voluntary exercise is known to increase hippocampal cAMP signaling (Shen et al., 2001); thus, it is also possible that changes in intracellular cAMP signaling, and subsequent protein kinase activity, could drive the up-regulation of hippocampal AEA content.

Despite the fact that both AEA and 2-AG are cognate ligands to the CB_1 receptor, it is not surprising that the two ligands responded differentially to VEx. AEA and 2-AG do not share common pathways of synthesis and metabolism, with 2-AG being largely produced by phospholipase C-mediated generation of diacylglycerol, which is subsequently converted to 2-AG by the actions of diacylglycerol lipase (Bisogno et al., 2005). Additionally, 2-AG is primarily metabolized by monacylglycerol lipase, while AEA is primarily metabolized by FAAH (Bisogno et al., 2005). As the pathways of synthesis and metabolism of these ligands are dissociable, it is not unexpected that VEx would evoke changes in one of these ligands and not the other.

Neural progenitor cells in the hippocampus express CB_1 and CB_2 receptors and synthesize both AEA and 2-AG (Aguado et al., 2005, 2006; Jiang et al., 2005; Molina-Holgado et al., 2007; Palazuelos et al., 2006). Activation of these receptors, presumably on the progenitor cells themselves, pushes these precursor cells into a mitogenic state to produce a progeny population. Several *in vitro* and *in vivo* studies have demonstrated that the CB_1 receptor can activate the phosphatidylinositol-3 kinase (PI3K)/Akt pathway (Galve-Roperh et al., 2002; Gomez del Pulgar et al., 2000; Molina-Holgado et al., 2002, 2005, 2007; Ozaita et al., 2007) and indicate that this pathway is instrumental for cannabinoid-induced proliferation (Molina-Holgado et al., 2007). Activation of the PI3K/Akt pathway within progenitor cells is sufficient to induce proliferation and is a common pathway through which many growth factors also induce proliferation (Aberg et al., 2003; Jin et al., 2005; Peltier et al., 2007). VEx can activate the PI3K/Akt pathway (Chen and Russo-Neustadt, 2005) although it remains to be determined if this signaling pathway is required for VEx-induced

proliferation. It should also be noted that endocannabinoids are likely not the sole mediators of the proliferatve effects of VEx; in particular, trophic factors, such as VEGF and IGF-1, have also been found to be essential for this phenomenon (Fabel et al., 2003; Trejo et al., 2001). Thus, the most parsimonious explanation is that endocannabinoids and neurotrophic factors act in concert to regulate VEx-induced cell proliferation; a hypothesis that seems quite feasible given the convergence in signal transduction pathways that are utilized by these systems (Aberg et al., 2003; Bouaboula et al., 1997; Galve-Roperh et al., 2002; Gomez del Pulgar et al., 2000; Jin et al., 2005; Molina-Holgado et al., 2002, 2005, 2007; Ozaita et al., 2007; Peltier et al., 2007).

At the clinical level, exercise has demonstrated therapeutic benefit for an array of psychiatric and neurological conditions, such as Alzheimer's disease and depressive illness (Cotman et al., 2007; Dunn et al., 2005; Ernst et al., 2006; Stevens and Killeen, 2006). We have previously demonstrated that endocannabinoid signaling is dampened in the hippocampus in an animal model of depression and increased following treatment with a conventional antidepressant (Hill et al., 2005, 2006a), whereas local infusions of a CB_1 receptor agonist directly into the dentate gyrus evoked an antidepressant-like response (McLaughlin et al., 2007). It has been suggested that induction of neural progenitor proliferation contributes to the antidepressant effects of exercise (Bjornebekk et al., 2005; Ernst et al., 2006). Therefore, the VEx-induced increase in hippocampal endocannabinoid activity could contribute to the antidepressant effects of this regimen, potentially via its contribution to changes in cell proliferation. Additionally, the neuroprotective effects of exercise might be afforded by increased endocannabinoid signaling. Both exercise and endocannabinoid signaling can dampen excitotoxic damage within the hippocampus and improve long-term outcomes following a neurological insult, indicating the possibility of functional overlap (Gobbo and O'Mara, 2005; Griesbach et al., 2004; Marsicano et al., 2003; Panikashvili et al., 2001). Future research should examine the extent to which endocannabinoid signaling contributes to the neuroprotective and antidepressant effects of exercise.

In conclusion, these data demonstrate that VEx enhances endocannabinoid signaling in the hippocampus through both effects on ligand availability and receptor sensitivity to agonist. Furthermore, this enhanced endocannabinoid signaling appears to contribute to the VEx-induced increase in cell proliferation in the dentate gyrus, as treatment with a CB_1 receptor antagonist attenuated this effect. These data add to an increasing body of evidence supporting the hypothesis that the endocannabinoid system contributes to experience-induced alterations in plasticity within the hippocampus.

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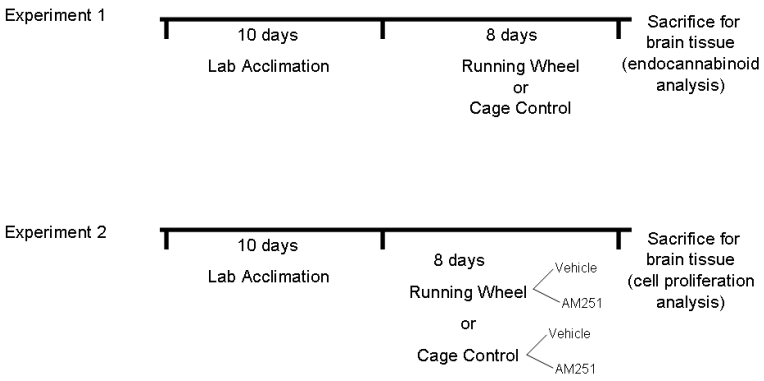

Figure 1. Flowchart of experimental procedures

Schematic representation of the housing/exercise protocol employed to generate tissue for examining the effects of voluntary exercise on the endocannabinois system (Experiment 1) and the role of endocannabinoid signaling in the effects of exercise on cell proliferation in the hippocampus (Experiment 2).

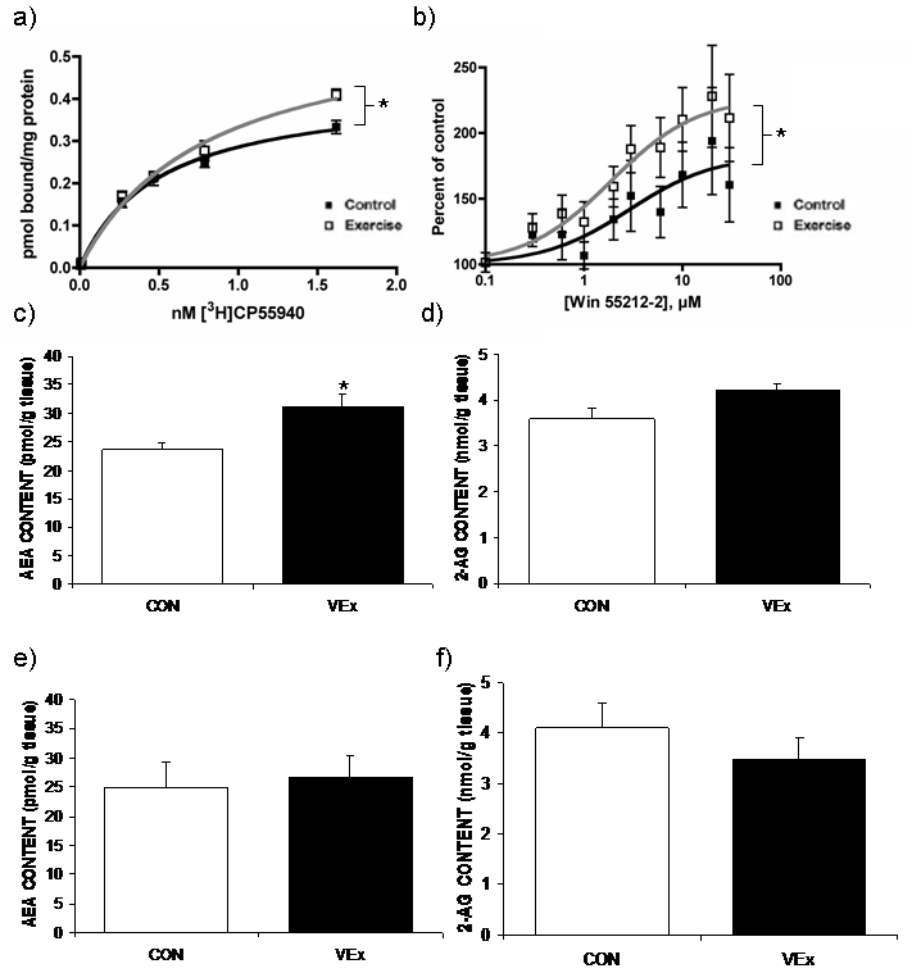

Figure 2. Voluntary exercise augments hippocampal endocannabinoid signaling

Eight days of voluntary exercise resulted in a significant increase in (a) CB_1 receptor binding site density in the hippocampus (n = 4-5 / condition), (b) CB_1 receptor mediated ^{35}S- GTPγS binding within the hippocampus (n = 4-5 / condition), and (c) tissue content of the endocannabinoid ligand anandamide (AEA) within the hippocampus (n = 7 / condition). There was no effect of voluntary exercise on (d) tissue content of the endocannabinoid ligand 2-arachidonoylglycerol (2-AG) in the hippocampus (n = 7 / condition), or (e) AEA content (n = 7 / condition) or (f) 2-AG content in the prefrontal cortex (n = 7 / condition). Values denoted are means ± SEM. * denotes significant differences ($p < .05$) between VEx animals and the control group (CON).

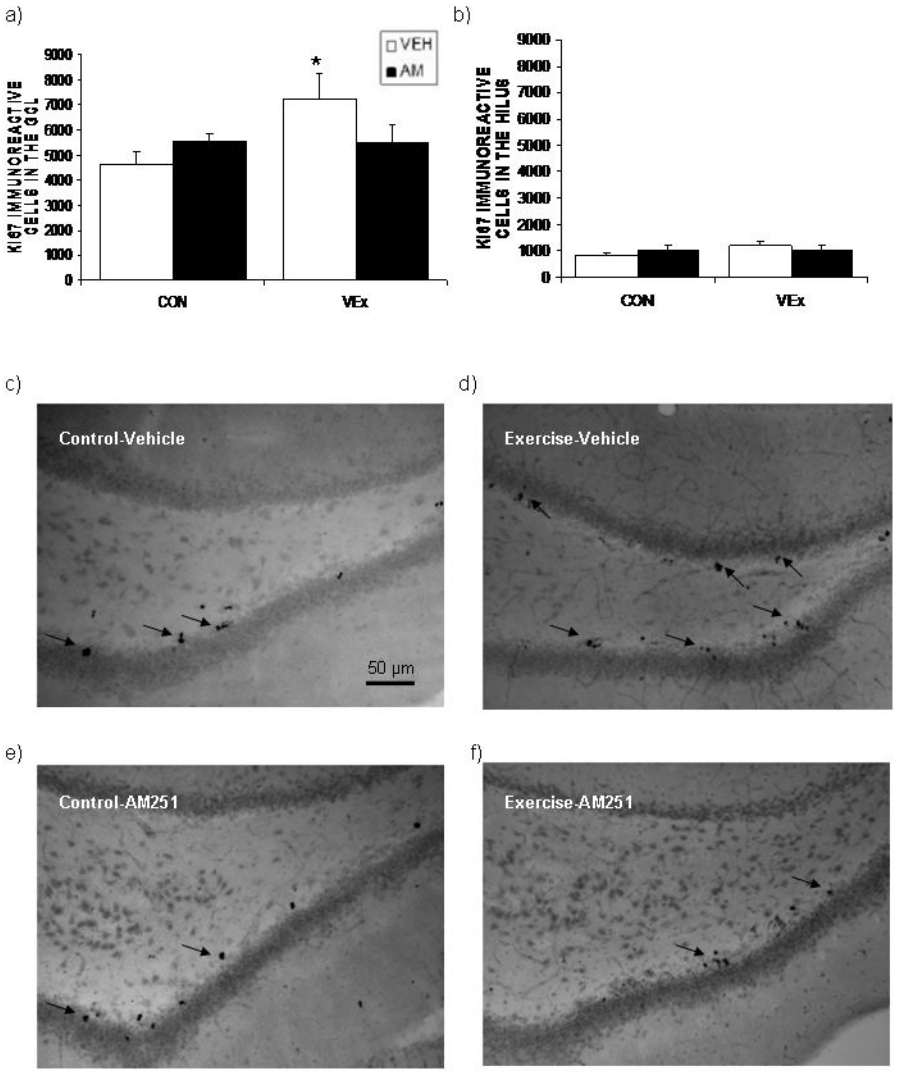

Figure 3. Antagonism of the cannabinoid CB_1 receptor attenuates the increase in cell proliferation in the dentate gyrus following voluntary exercise

(a) Animals which engaged in voluntary exercise (VEx) exhibited a significant increase in the total estimated number of proliferating cells expressing the endogenous cell cycle protein Ki-67 within the neurogenic region of the granule cell layer (GCL) of the dentate gyrus. This phenomenon was not seen following concurrent administration of the cannabinoid CB_1 receptor antagonist AM251 (AM; 1 mg/kg), which alone had no effect on cell proliferation. (b) Neither treatment, alone nor in combination, had any effect on the cellular expression of Ki-67 within the hilus of the dentate gyrus. Values denoted are means ± SEM; n = 5-6 / condition. * denotes significant differences ($p < .05$) between a treatment condition and the control group (CON) receiving vehicle injections (VEH). Representative photomicrographs (c, d, e, f) of the effects of voluntary exercise, AM251 administration (1 mg/kg), or both treatments combined on the immunoreactivity of Ki-67 (denoted by arrows) in the dentate gyrus at 10× magnification.

Table 1

The effects of voluntary exercise (VEx) on the maximal binding (B_{max}) and dissociation constant (K_d) of ^{3}H-CP55940, a cannabinoid CB_1 receptor agonist, from the CB_1 receptor and CB_1 receptor-mediated ^{35}S- GTPγS binding evoked by the CB_1 receptor agonist WIN 55,212-2.

Animals which engaged in voluntary exercise (VEx) exhibited a significant increase in the B_{max} and K_d of the CB_1 receptor in the hippocampus, that was accompanied by a significant reduction in the EC50 and a tendency for increased maximal stimulation (E_{max}) of CB_1 receptor-mediated ^{35}S- GTPγS binding in the hippocampus. There was no effect of VEx on CB_1 binding parameters within the prefrontal cortex. CB_1 receptor binding assays were performed in triplicate for each concentration of ^{3}H-CP55940 within each sample; GTPγS binding assays were performed in quadruplicate for each concentration of WIN 55,212-2 within each sample. Values denoted are means ± SEM. * denotes significant differences ($p < 0.05$) between VEx animals (n = 4-5) and the control group (CON; n = 4-5).

	CON	VEx
Hippocampus		
B_{max} (pmol/mg protein)	0.44 +/- 0.03	0.60 +/- 0.02*
K_d (nM)	0.54 +/- 0.08	0.82 +/- 0.06*
E_{max} (% baseline)	152.0 +/- 4.5	232.3 +/- 31.0
EC50 (nM)	4708.3 +/- 586.7	2240.6 +/- 396.1*
Prefrontal Cortex		
B_{max} (pmol/mg protein)	0.38 +/- 0.06	0.34 +/- 0.03
K_d (nM)	0.51 +/- 0.06	0.60 +/- 0.09

Table 2

The effects of voluntary exercise (VEx) on the maximal hydrolytic activity (V_{max}) and the binding affinity (K_m) of fatty acid amide hydrolase for anandamide. There was no effect of VEx on either the V_{max} or the K_m of FAAH in the hippocampus. For both treatment conditions, n = 5. Data are presented as means +/- SEM.

	CON	VEx
Hippocampus		
V_{max} (pmol/mg protein)	619.4 +/- 56.9	556.7 +/- 99.4
K_m (nM)	0.53 +/- 0.09	0.61 +/- 0.09

Table 3

The effects of voluntary exercise (VEx) and administration of the cannabinoid CB_1 receptor antagonist AM251 (1 mg/kg) on the volume of the granule cell layer (GCL) and hilus of the dentate gyrus.
There was no effect of VEx or AM251 on the volume of the GCL or the hilus of the dentate gyrus. For all treatment conditions, n = 5-6. Data are presented as means +/- SEM.

	CON	VEx
GCL (mm^3)		
Vehicle	3.08 +/- 0.12	3.19 +/- 0.13
AM251	3.14 +/- 0.18	3.28 +/- 0.29
Hilus (mm^3)		
Vehicle	7.16 +/- 0.29	7.27 +/- 0.58
AM251	7.81 +/- 0.57	7.12 +/- 0.12