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COUPLING ENERGY METABOLISM WITH A MECHANISM TO SUPPORT BRAIN-DERIVED NEUROTROPHIC FACTOR-MEDIATED SYNAPTIC PLASTICITY

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Abstract—Synaptic plasticity and behaviors are likely dependent on the capacity of neurons to meet the energy demands imposed by neuronal activity. We used physical activity, a paradigm intrinsically associated with energy consumption/expenditure and cognitive enhancement, to study how energy metabolism interacts with the substrates for neuroplasticity. We found that in an area critical for learning and memory, the hippocampus, exercise modified aspects of energy metabolism by decreasing oxidative stress and increasing the levels of cytochrome c oxidase-II, a specific component of mitochondrial machinery. We infused 1,25-dihydroxyvitamin D3, a modulator of energy metabolism, directly into the hippocampus during 3 days of voluntary wheel running and measured its effects on brain-derived neurotrophic factor-mediated synaptic plasticity. Brain-derived neurotrophic factor is a central player for the effects of exercise on synaptic and cognitive plasticity. We found that 25-dihydroxyvitamin D3 decreased exercise-induced brain-derived neurotrophic factor but had no significant effect on neurotrophin-3 levels, thereby suggesting a level of specificity for brain-derived neurotrophic factor in the hippocampus. 25-Dihydroxyvitamin D3 injection also abolished the effects of exercise on the consummate end-products of brain-derived neurotrophic factor action, i.e. cyclic AMP response element-binding protein and synapsin I, and modulated phosphorylated calmodulin protein kinase II, a signal transduction cascade downstream to brain-derived neurotrophic factor action that is important for learning and memory. We also found that exercise significantly increased the expression of the mitochondrial uncoupling protein 2, an energy-balancing factor concerned with ATP production and free radical management. Our results reveal a fundamental mechanism by which key elements of energy metabolism may modulate the substrates of hippocampal synaptic plasticity.

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Abbreviations: ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; CAMKII, calmodulin protein kinase II; COX-II, cytochrome oxidase-II; C_T, threshold cycle; cytC, cytochrome C; DNPH, dinitrophenylhydrazine; D3, 1α,25-dihydroxyvitamin D3; exc, exercise control; exc/D3, exercise/1α,25-dihydroxyvitamin D3; GAPDH, glyceraldyehyde-3-phosphate dehydrogenase; OS, oxidative stress; p-CREB, phosphorylated CREB; RT-PCR, reverse transcription polymerase chain reaction; sed, sedentary control; S.E.M., standard error of the mean; slm, stratum lacunosum moleculare; UCP2, uncoupling protein 2; UNG, uracil glycosylase.

Key words: metabolism, exercise, CREB, synapsin I, hippocampus, mitochondria.

Emerging evidence indicates that events associated with energy balance can impact synaptic and cognitive function (Wu et al., 2004; Liu et al., 2002). Neuronal and cognitive plasticity rely on the management of cellular energy and are therefore influenced by experiences that intrinsically affect the energy status of the cell, such as physical activity (Mattson et al., 2004; Lee et al., 2000, 2002). Physical activity has been shown to enhance cognitive function in both humans and animals (Fordyce and Wehner, 1993; Kramer et al., 1999; Laurin et al., 2001). A central player found to mediate the effects of exercise on synaptic and cognitive plasticity is brain-derived neurotrophic factor (BDNF; Molteni et al., 2004; Vaynman et al., 2003, 2004). It has been found that in the hippocampus, an area critical for supporting cognitive function, inhibiting the action of BDNF during exercise abrogates the exercise-induced enhancement in learning and memory, and reduces the expression of molecules involved in synaptic plasticity and cognition (Vaynman et al., 2004). BDNF facilitates synaptic plasticity. *In vivo* BDNF infusion induces a long-lasting potentiation of perforant path–dentate gyrus connections (Messaoudi et al., 1998). Transgenic animals with diminished BDNF expression lose the ability to induce long-term potentiation (Patterson et al., 1996), and hippocampal BDNF reinstitution amends this deficit (Patterson et al., 1996; Korte et al., 1995).

In addition to its role in synaptic plasticity and cognition, BDNF is involved in controlling energy metabolism. BDNF addition increases mitochondrial activity (El Idrissi and Trenker, 1999). In turn, heightened oxidative stress (OS), the harmful by-product of mitochondrial respiration, decreases BDNF levels (Wu et al., 2004). The importance of BDNF in impacting energy metabolism is seen in disorders of energy balance, such as obesity, insulin insensitivity, and hyperglycemia. Mice with reduced BDNF levels are obese and hyperglycemic (Kernie et al., 2000; Lyons et al., 1999). Peripheral or central BDNF administration reduces body weight, normalizes glucose levels in diabetic rodents (Tonra et al., 1999), and increases insulin sensitivity (Pelleymounter et al., 1995; Nakagawa et al., 2000).

Given the involvement of BDNF in energy balance and synaptic plasticity, a central question is whether alterations in energy balance can impact aspects of synaptic plasticity modulated by BDNF. A possible answer may lie within the mitochondrial powerhouse of the cell, whose functions

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likely extend to meet the energy demands imposed by activity on the synapse. Particularly, the uncoupling protein 2 (UCP2), a member of the superfamily of uncoupling proteins located in the mitochondrial inner membrane, may support these necessary molecular mechanisms to impact the synapse (Andrews et al., 2005). UCP2 functions to leak hydrogen protons from the intermembrane space to the mitochondrial matrix, thereby uncoupling substrate oxidation from ATP synthesis (Bouillaud et al., 1985; Boss et al., 1997; Vidal-Puig et al., 1997; Mao et al., 1999; Sanchis et al., 1998). UCP2 belongs to a family of uncoupler proteins that have been identified to date, comprising UCP1-3, UCP4 and BMCP1 (Lengacher et al., 2004; Arsenijevic et al., 2000; Diano et al., 2000; Horvath et al., 1999). UCP2 is abundantly expressed in the hippocampus (Richard et al., 2001; Diano et al., 2003) and specifically in select neuronal populations involved with the coordination of homeostasis and subject to massive neuronal and humoral regulation (Horvath et al., 1999; Diano et al., 2000). These discoveries have engaged the intriguing possibility that UCP2 may function to modulate the events by which energy supply and consumption interface with the substrates for neuroplasticity in the brain. Thus, the ability of behaviors such as exercise to influence neural plasticity may lie in their capacity to access the mitochondrial machinery critical for maintaining cellular energy demands and metabolism. In these studies, we investigated whether physical activity uses specific aspects of energy metabolism to alter BDNF-mediated plasticity in the hippocampus.

EXPERIMENTAL PROCEDURES

Exercise paradigm

Adult male Sprague–Dawley (Charles River, Wilmington, MA, USA) rats (3 months of age) were randomly assigned to sedentary or exercise groups ($N=6$ animals per group). We used three exercise groups and a sedentary ($N=6$ rats per group) to perform our blocking experiments using 3 days of exercise. To get a better understanding of how exercise affects mitochondrial metabolism, we measured UCP2 expression in the hippocampus after 3, 7, and/or 28 days of exercise. All rats were individually housed in standard polyethylene cages in a 12-h light/dark cycle at 22–24 °C, with food and water *ad libitum*. The exercise rats were given access to a wheel (diameter=31.8 cm, width=10 cm) that freely rotated against a resistance of 100 g attached to a receiver that monitored revolutions every hour (VitalViewer Data Acquisition System software, Mini Mitter Company, Inc., Sunriver, OR, USA). The control rats were confined to a cage with no access to a wheel. We chose a voluntary exercise paradigm because it simulates aspects of human behavior in which animals choose how much to run. Although voluntary wheel running produces a range in running behavior which could be controlled for in a forced treadmill paradigm, we have successfully found that by using a voluntary wheel running paradigm we can correlate running distance with changes in molecular markers of synaptic plasticity in the hippocampus in individual animals (Vaynman et al., 2003). The exercise rats were given three nights of wheel running. All rats were killed and brain tissue was collected the morning after the last running period. For biochemical assays, the animals were killed by decapitation and their hippocampi were rapidly dissected out, immediately placed on dry ice, and stored at −70 °C. All procedures were approved by UCLA animal Research Committees and followed the guidelines of the American Physiological Society of Animal Care.

Drugs

We used 1α,25-dihydroxyvitamin D3 (D3) obtained from Biomol, Plymouth, PA, USA, which has been shown to disrupt mitochondrial metabolism (Weitsman et al., 2003) and inhibit the expression of the mitochondrial uncoupler UCP2 (Shi et al., 2002). As previously described in Shi et al. (2002), we used 1 nM of D3 to block UCP2 expression. We used cytochrome C (cytC), obtained in powder from Sigma, St. Louis, MO, USA, as the control drug since it has been used as a standard control for microbead injections (Vaynman et al., 2003, 2004; Lom and Cohen-Cory, 1999). CytC was prepared in accordance to previously described methods (Vaynman et al., 2004).

Preparation of microbeads

We administered all drugs via injection of fluorescent latex microbeads (Lumafluor, Naples, FL, USA) directly into the right hippocampus, resulting in a consistent and effective blockade of targets as shown in the results. Infusion of drugs was achieved by coupling them to microbeads, which we have previously used as a reservoir for the successful delivery of viable inhibitors into the hippocampus (Vaynman et al., 2003, 2004). We prepared microbeads by the methods described previously (Riddle et al., 1997; Lom and Cohen-Cory 1999). Briefly, these consisted of coating the microbeads with each drug via passive absorbency by incubating overnight at 4 °C with a 1:5 mix of microbeads to D3 (1 nM, Shi et al., 2002) and cytC (100 ng/μl in sterile water; Lom and Cohen-Cory 1999). The morning after coating the microbeads, the solution was centrifuged at 14,000×*g* for 30 min and the microbeads were resuspended in sterile water at a 10% concentration.

Injection of drugs into the hippocampus

We randomly divided rats into 3-day exercise (exc) and sedentary control (sed) groups, pre-treated with D3 or the standard control (Lom and Cohen-Cory, 1999), to generate a total of four groups ($N=6$ rats per group); exc, exercise/D3 (exc/D3), sed, and sedentary/D3 (sed/D3). All rats received the injection once prior to the 3-day running period, administered early in the morning, thereby providing an ample recovery time for all animals to begin running that same evening. We used a unilateral injection to the right hippocampus to be consistent with previous blocking experiments, which have shown that using a unilateral injection to the right hippocampus to block the action of BDNF is sufficient to abolish the exercise-induced enhancement in synaptic plasticity markers and learning and memory (Vaynman et al., 2003, 2004). All measurements were derived from tissues extracted from the right hippocampus. We did not use the contralateral hippocampus as a control since a unilateral injection can cause changes on the contralateral side due to connecting fibers (Amaral and Witter, 1989). All animals were anesthetized by isoflurane (2–2.5%) utilizing the Mobile Laboratory Animal Anesthesia System, and positioned in a stereotaxic apparatus that was used to secure the animal and to measure the sight for injection. D3 or the standard control solution imbedded in microbeads was injected into the right hippocampus (3.8 mm posterior to Bregma, 1 mm from the midline, and 3.7 mm vertically) using a Hamilton syringe in a volume of 2 μl over 15 min. Previous studies by Quattrochi et al. (1989), Riddle et al. (1995, 1997), and Lom and Cohen-Cory (1999) have reported the successful delivery by microbeads of bioactive agents such as neurotrophins and neurotransmitter agonists/antagonists into highly localized brain regions. The location of microbead injection was verified by histological examination of selected brains, as previously described (Quattrochi et al., 1989; Riddle et al., 1995). We visually inspected all the brains at the time of dissection, such that only those showing characteristic markings of microbeads of the right hippocampus were used for mRNA

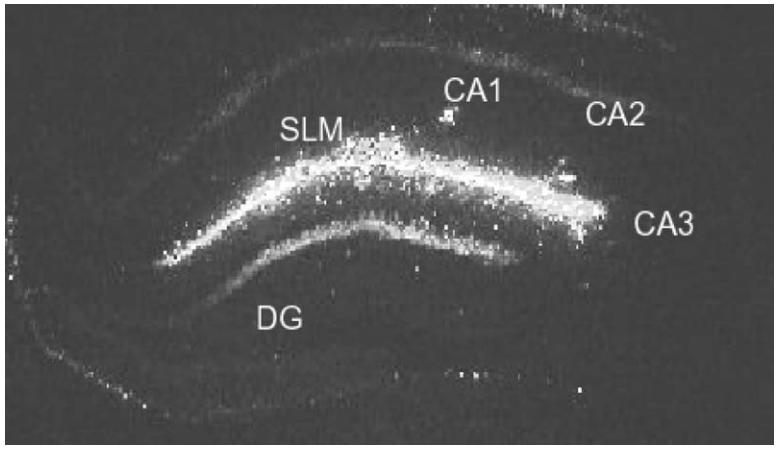

Fig. 1. Microbead injection. Using fluorescence microscopy, tissue section in the saggital plane shows that the site of microbead injection is concentrated in the slm of the right hippocampus. For convenience, hippocampal areas CA1, CA2, CA3, and dentate gyrus (DG) have been labeled.

measurements. The microbead injection site was additionally verified by florescence microscopy using an Olympus BX51 microscope (Fig. 1), such that it corresponded to the site of microbead injection concentrated in the stratum lacunosum moleculare (slm) used in our previous studies examining BDNF-mediated synaptic plasticity and cognition in the hippocampus (Vaynman et al., 2003, 2004).

Measurement of oxidized proteins

The amount of oxidized proteins containing carbonyl groups was measured by using Oxyblot kit (Intergen, Purchase, NY, USA). In brief, the protein sample (10 μg) was reacted with 1$\times$ dinitrophenylhydrazine (DNPH) for 15 min, and then neutralized with a solution containing glycerol and β-mercaptoethanol. Following, samples were electrophoresed on an 8% polyacrylamide gel and electrotransferred onto a nitrocellulose membrane. After membranes were blocked, they were incubated overnight with a rabbit DNPG antibody (1:150) at 4 °C, followed by incubation in goat anti-rabbit (1:300) for 1 h at room temperature. After rinsing with buffer, immunocomplexes were visualized by chemiluminescence using the ECL kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) according to the manufacturer's instructions. The film signals were digitally scanned and then quantified using NIH Image software.

Isolation of total RNA and real-time quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated using RNA STAT-60 kit (TEL-TEST, Inc., Friendswood, TX, USA) as per manufacturer's protocol. Quantification was carried out by absorption at 260 nm. The mRNAs for UCP2, BDNF, NT-3, CREB and synapsin I were measured by real-time quantitative RT-PCR using PE Applied Biosystems prism model 7700 sequence detection instrument, which directly detects the RT-PCR product without downstream processing. This is achieved by monitoring the increase in fluorescence of a dye labeled DNA probe, one that is specific for the factor of interest plus another specific for glyceraldyehyde-3-phosphate dehydrogenase (GAPDH) gene, which has been previously used as a successful endogenous assay control (Molteni et al., 2002; Griesbach et al., 2002). Total RNA (100 ng) was converted into cDNA using TaqMan EZ RT-PCR Core reagents (Perkin-Elmer, Branchburg, NJ, USA). The sequences of probes, forward and reverse primers, designed by Integrated DNA Technologies (Coralville, IA, USA) were: UCP2: (5′-TATCTC-CGACCACCGCCAGCC CG-3′); forward: (5′-ATGTGGTAAAGGTC-CGCTTCC-3′); reverse: (5′-GTA GCC TTCGACAGTGCTCTG-3′); BDNF: (5′-AGTCATTTGCGCACAACTTTAAAAGTCTGCATT-3′); forward: (5′ -GGACATATCCATGACCAGAAAGAAA-3′); reverse: (5′-GCAACAAACCACAACATTATCGAG-3′); CREB: (5′-CATGGCAC-GTAATGGAGACTACCGCA-3′); forward: (5′-CCGCCAGCATGCC-TTC-3′); reverse: (5′-TGCAGCCCAATGACCAAA-3′); synapsin I: (5′-CATGGCACGTAATGGAGACTACCGCA-3′); forward: (5′-CCGC-CAGCATGCCTTC-3′); reverse: (5′-TGCAGCCCAATG ACCAAA-3′); NT-3: (5′-TGACCGACAAGTCCTCAGCCATTGAC-3′); forward: (5′-TGTGACAGTGAGAGCCTGTGG-3′); reverse: (5′-TGTAACCTGGT-GTCCCCGAA-3′). The endogenous control probe, specific for the GAPDH gene, served to standardize the amount of RNA sample and consisted of the following oligonucleotide sequence (5′-CCGACTCTTGCCCTTCGAAC-3′). The RT-reaction steps consisted of an initial 2 min incubation step at 50 °C to activate uracil glycosylase (UNG) and were followed by 30 min of reverse transcription at 60 °C. A completion step for UNG deactivation was performed for 5 min at 95 °C. The 40 cycles of two-step PCR-reaction consisted of a 20 s period at 94 °C and a 1 min period at 62 °C.

Protein measurements

Hippocampal extracts were prepared in lysis buffer (137 mM NaCl, 20 mM Tris–HCl pH 8.0, 1% NP-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 1 μg/ml leupeptin, 0.5 mM sodium vanadate). Homogenates were centrifuged to remove insoluble material (12,000 r.p.m. for 20 min at 4 °C) and the total protein concentration was determined according to the MicroBCA procedure (Pierce, Rockford, IL, USA). BDNF protein was quantified using an enzyme-linked immunosorbent assay (ELISA; BDNF Emax ImmunoAssay system Kit; Promega Inc., Madison, WI, USA) as per manufacturer's protocol.

The homogenates were centrifuged, the supernatants were collected and total protein concentration was determined according to the Micro BCA procedure (Pierce), using bovine serum albumin as a standard. Equal amounts (25 μg) of protein samples were separated by electrophoresis on a 10% polyacrylamide gel and electrotransferred to a nitrocellulose membrane. Non-specific binding was blocked with 5% nonfat milk. Synapsin I, CREB, p-CREB, and p-calmodulin protein kinase II (CAMKII) proteins were analyzed by Western blot as previously described (Gomez-Pinilla et al., 2001), quantified by densitometric scanning of the film under linear exposure conditions and normalized for actin levels. Membranes were incubated with the following primary antibodies: anti-synapsin I (1:2000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-total CREB (1:2000; Cell Signal-

ing Technology, Inc., Beverly, MA, USA), anti-phosphorylated CREB (p-CREB; 1:2000; Cell Signaling Technology, Inc.), anti-cytochrome oxidase-II (COX-II; 1:1000, Santa Cruz Biotechnology), anti-phosphorylated CAMKII (p-CAMKII; 1:5000; Santa Cruz Biotechnology), and anti-actin (1:2000; Santa Cruz Biotechnology) followed by anti-goat IgG horseradish peroxidase conjugate for synapsin I, COX-II, and actin or anti-rabbit IgG horseradish peroxidase conjugate for total CREB, p-CREB, p-CAMKII (Santa Cruz Biotechnology). After rinsing with buffer, immunocomplexes were visualized by chemiluminescence using the ECL kit (Amersham Pharmacia Biotech Inc.) according to the manufacturer's instructions. The film signals were digitally scanned and then quantified using NIH Image software.

Statistical analyses

We used GAPDH and actin as internal controls for RT-PCR and Western blotting, respectively, as exercise did not alter their expression (Molteni et al., 2002). Quantification of the TaqMan RT-PCR results was performed by plotting fluorescent signal intensities against the number of PCR cycles on a semilogarithmic scale. A threshold cycle (C_T) was designated as the amplification cycle at which the first significant increase in fluorescence occurred. The C_T value of each sample was compared with that of the internal standard. These processes were fully automated and carried out using the ABI sequence detector software version 1.6.3 (PE Biosystem). Taqman EZ RT-PCR values for UCP2, BDNF, CREB, synapsin I, and NT-3 were corrected by subtracting values for GAPDH as previously described (Molteni et al., 2002; Griesbach et al., 2002). These corrected values were used to make cross-group comparisons. The mean values for the mRNA and protein levels were computed for the sedentary and exercise groups and compared using an analysis of variance (ANOVA) with repeated measures. We analyzed the biochemical data with exercise and drug applications as the independent factors and UCP2, OS, COXII, BDNF, synapsin I, CREB, and CAMKII as the dependent variables. A Fischer-test was used for cross-group comparisons. Results were expressed as the mean percent of control values for graphic clarity and represent the mean±standard error of the mean (S.E.M.).

RESULTS

Effect of exercise and D3 infusion on OS

The mitochondria work constantly to supply energy for neuronal processes by using oxidative phosphorylation. A normal by-product of mitochondrial respiration is free radical formation. When an imbalance occurs between the production of free radicals and the ability of cells to guard against them it is commonly referred to as OS (Ebadi et al., 1996; Jenner and Olnaw, 1996; Simonian and Coyle, 1996). We wanted to examine the possibility that exercise can limit the amount of OS occurring in the hippocampus by employing mitochondrial metabolism. Therefore, we assessed oxidized protein levels as markers of OS (Wu et al., 2004) under D3 infusion following exercise. The levels of oxidized proteins were measured using Western Blot analysis, in which carbonyl groups on oxidized proteins were derivatized with DNPH and detected by using a DNPH antibody. We found that exercise significantly ($P<0.05$) reduced the levels of OS (76±6%) below sed levels (100±6%; exc vs. sed controls; Fig. 2). D3 application significantly abrogated the exercise-induced reduction in OS levels; exc/D3 animals showed higher OS levels (130±20%) than both the exercise and the sed groups and with a value that approached that produced by D3 infusion in the sedentary group (142±13%, exc/D3 vs. sed/D3; Fig. 2).

Exercise increases COXII levels in the hippocampus

In the hippocampus, it has been demonstrated that decreases in the amount of OS incurred by cells are accompanied by increases in mitochondrial number (Diano et al., 2003). We found that exercise significantly ($P<0.05$) increased the amount of COX-II (125±6%) above sed animals

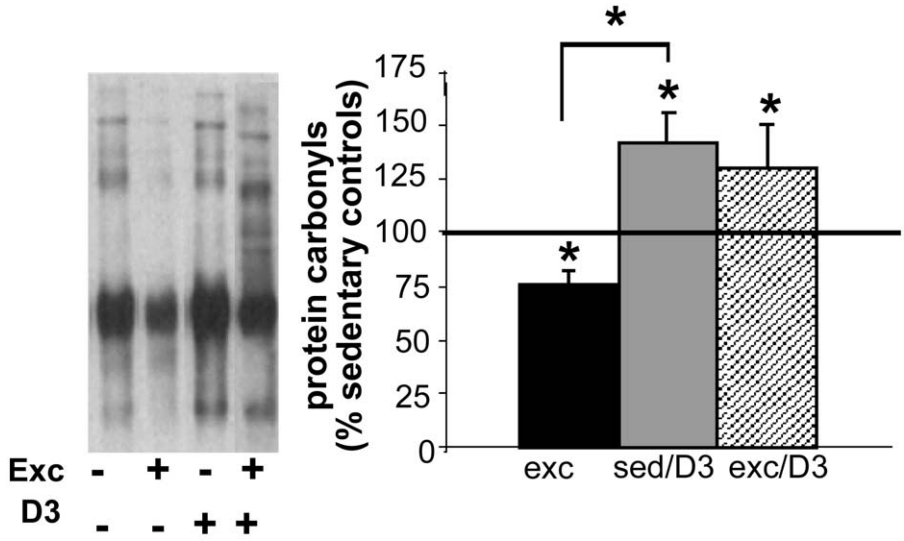

Fig. 2. The effects of exercise and D3 on oxidized protein levels in the hippocampus. Measurement of oxidized protein levels in the hippocampus was determined by using the Oxyblot kit. There was a significant effect of exercise, such that oxidized protein levels were lower in exercising animals compared with those of the sed group. D3 infusion in both the sedentary and exercise conditions increased the levels of oxidized proteins above sed levels. Representative sample of the Oxyblot from hippocampal tissue of each group is shown to the left of the graph. Results are displayed as percentages of sed levels (represented by the 100% line). Each value represents the mean±S.E.M. (ANOVA, Fischer's test, * $P<0.05$).

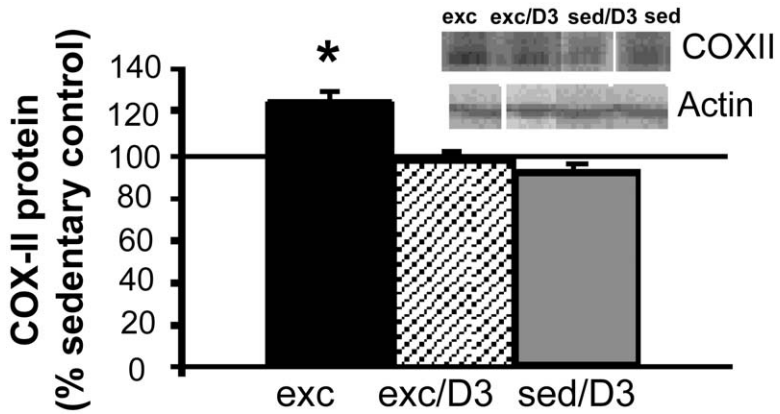

Fig. 3. D3 infusion modulates COX-II in the hippocampus. Exercise significantly increased COX-II protein levels above sed levels. D3 infusion during exercise abrogated the exercise-induced increase in COX-II protein levels. D3 had no significant effect COX-II protein levels in sedentary animals. Representative immunoblots are shown in the order of group presentation overlaid on the actin controls for each group. Furthermost right immunoblot represents sed levels. Results are displayed as percentages of sed levels (represented by the 100% line). Each value represents the mean±S.E.M. (ANOVA, Fischer's test, * $P<0.05$).

(100±8%), while D3 abrogated this exercised induced increase (96±5%; exc/D3). Application of the inhibitor had no significant effect on COX-II levels in sedentary animals (91±4%, sed/D3; Fig. 3).

Effect of exercise and D3 infusion on BDNF in the hippocampus

To determine the possibility that energy metabolism may modulate BDNF-mediated synaptic plasticity, we measured BDNF levels in the hippocampus under D3 infusion. We found that while exercise significantly ($P<$ 0.05) increased the mRNA levels of BDNF (134±7%) in the hippocampus above sed, D3 fully blocked the exercise-induced effect on BDNF mRNA (134±7% to 111±14%; Fig. 4A), effectively reducing them to the mRNA levels of sed (100±5%, exc/D3 vs. sed; Fig. 4A). We found that D3 did not have an effect on BDNF mRNA levels in the sedentary condition, such that there was no significant difference between the sed/D3 (103±9%) and the sed group (100±5%, Fig. 4A). We found that exercise significantly ($P<0.05$) increased BDNF protein levels (129±9%) above sed levels (100±7%; Fig. 4B). D3 fully blocked the exercise-induced effect on BDNF protein levels (129±9% to 109±5%), by reducing them to sed levels (100±7%, exc/D3 vs. sed; Fig. 4B). D3 infusion in sedentary animals did not significantly affect BDNF protein levels (116±8%, sed/D3 vs. sed; Fig. 4B).

To test the possibility that D3 has a level of specificity for BDNF, we measured NT-3 expression under exercise and D3 infusion. Previously, we measured NT-3, another neurotrophin expressed in hippocampal neurons, to show that exercise specifically acts to increase BDNF and not NT-3 expression (Vaynman et al., 2003). We found that exercise did not significantly increase the expression of NT-3 (110±10%) above sed levels. Application of D3 did not significantly alter NT-3 expression in exercising animals (82±7%) as compared to the sed group. D3 infusion did not significantly affect NT-3 levels in sedentary animals (105±10%; Fig. 4C).

Effect of exercise and D3 infusion on plasticity markers; CREB and synapsin I

To evaluate whether metabolism associated with exercise modulates the end-products of BDNF action on vesicular release and transcriptional mechanisms, we measured synapsin I and CREB levels, respectively, under D3 infusion during exercise. BDNF influences synaptic transmission by modulating the efficacy of neurotransmitter release (Kang and Schuman, 1996; Bolton et al., 2000) and stimulating the synthesis of vesicle-associated proteins (Schinder et al., 2000) and the dynamics of gene expression by regulating transcriptional factors (Finkbeiner et al., 1997; Tully, 1997). Previously we have shown that exercise uses BDNF to regulate two of its consummate end-products on synaptic transmission and gene regulation, i.e. synapsin I, and CREB, respectively (Vaynman et al., 2003, 2004). Synapsin I is a terminal specific protein, recently implicated in learning (Garcia et al., 2004) and involved in synaptic vesicle clustering and release (Jovanovic et al., 2000) and the formation and maintenance of the presynaptic structure (Melloni et al., 1994; Takei et al., 1995). CREB is one of the best-described stimulus-induced transcriptional regulators, involved in adaptive responses (Finkbeiner et al., 1997; Finkbeiner, 2000) and long-term memory formation (Dash et al., 1990; Bourtchouladze et al., 1994; Tully et al., 1994; Yin et al., 1995; Frank and Greenberg, 1994).

Exercise significantly ($P<0.05$) increased the mRNA levels of CREB (130±4%) above sed levels (100±5%; Fig. 5A). D3 completely blocked the effect of exercise on CREB mRNA levels, reducing them to those of the sed group (130±4% to 87±6%, exc vs. exc/D3; Fig. 5A). D3 infusion had no significant effect on CREB mRNA levels in sedentary animals (92±4%, sed/D3; Fig. 6A). We found that exercise significantly ($P<0.05$) increased the levels of CREB protein (144±18%) in the exc group above those of the sed group (100±11%; Fig. 5B). D3 infusion was sufficient to completely abrogate the exercise-induced increase in CREB (144±18%

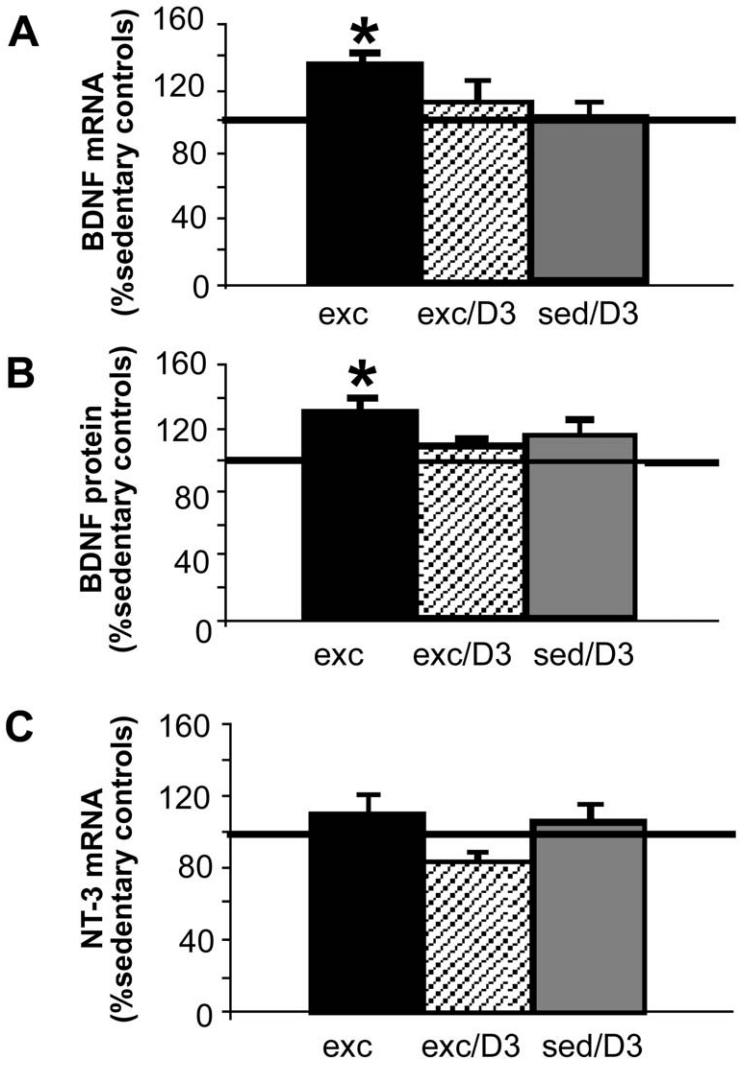

Fig. 4. D3 infusion modulates BDNF in the hippocampus. Exercise significantly increased the mRNA and protein levels of BDNF above sed levels (A and B respectively). D3 infusion during exercise abrogated the exercise-induced increase in BDNF mRNA and protein levels, reducing them to the state of the sed group (A, B). Application of D3 had no significant effect on BDNF mRNA and protein I levels in sedentary animals. Results are displayed as percentages of sed levels (represented by the 100% line). Each value represents the mean±S.E.M. (ANOVA, Fischer's test, * $P<0.05$).

to 106±7%, exc vs. exc/D3) in exc/D3 rats; thereby effectively reducing them to sed levels (Fig. 5B). D3 application seemed to have no effect during the sedentary condition, as CREB levels (97±5%) in the sed/D3 group did not significantly differ from those of the sed group (Fig. 5B).

We measured p-CREB to get a better indication of its active state. We found that exercise significantly ($P<0.05$) increased p-CREB levels (146±13%) in the exc group above sed levels (100±5%; Fig. 5C). D3 infusion into exercising animals fully blocked the exercised-induced increase in p-CREB levels (146±13% to 93±3%; exc vs. exc/D3, respectively). D3 did not decrease p-CREB levels in the sedentary condition, such that p-CREB levels of the sed/D3 rats (98±8%) were not significantly different from those of the sed group (Fig. 5C).

We examined synapsin I, an important component for determining the size of the synaptic vesicle reserve pool (Li et al., 1995; Takei et al., 1995), which serves to sustain neurotransmitter release under high frequency stimulation (Pieribone et al., 1995). In our previous studies we have found that exercise increases synapsin I expression (Vaynman et al., 2003, 2004). Exercise significantly ($P<0.05$) increased the mRNA levels of synapsin I (139±6%) above sed levels (Fig. 6A). The application of D3 significantly ($P<0.05$) increased synapsin I mRNA levels in sedentary animals (142±5%; sed/D3) above those of the sed group. Exercise with D3 injection further increased synapsin I mRNA levels (158±9%; exc/D3) above sed levels (Fig. 6A). We found that exercise also significantly ($P<0.05$) increased the protein levels of synapsin I (140±17%) above sed levels (100±12%). D3 infusion abrogated the exercise-induced increase in synapsin I levels; thereby reducing them to sed levels (140±17% to 100±5%, exc vs. exc/D3). D3 seemed to have no significant effect on synapsin I level in sedentary animals (108±3%, sed/D3; Fig. 6B).

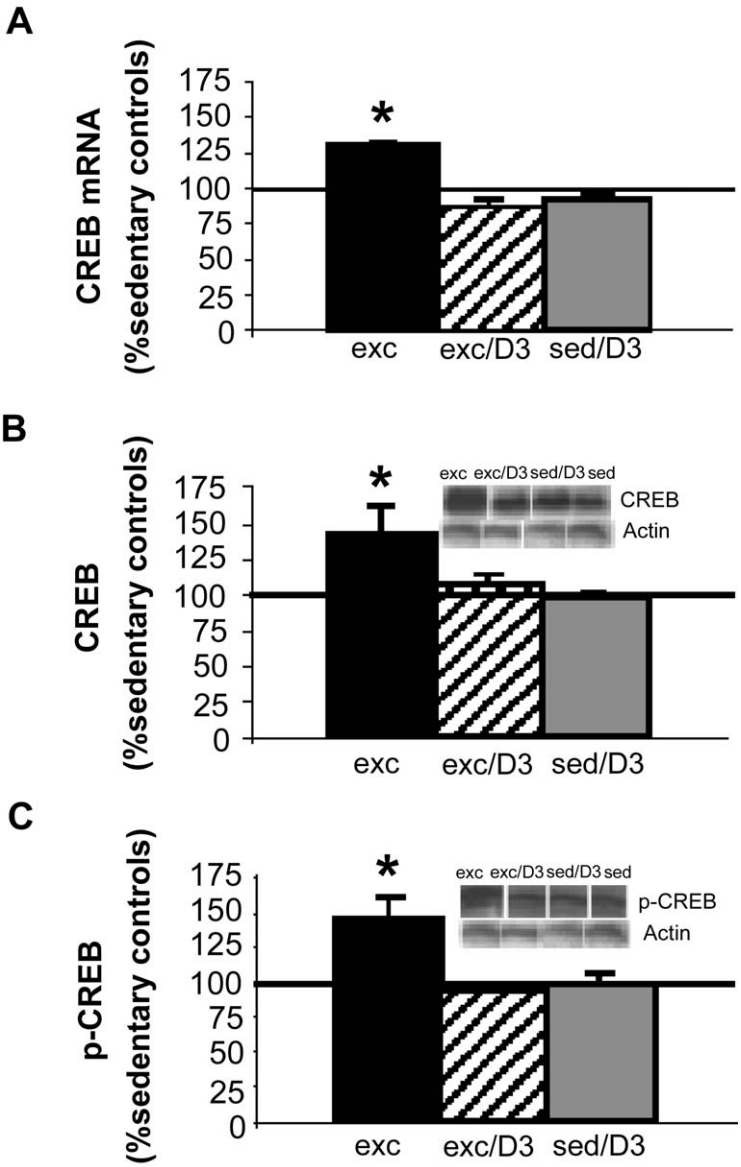

Fig. 5. D3 infusion modulates CREB in the hippocampus. Exercise significantly increased CREB mRNA (A) and protein levels (B) and the active form of CREB, phospho-CREB (C), above sed levels. Infusing D3 during exercise abrogated the exercise-induced increase in CREB mRNA and protein (total CREB and phospho-CREB). D3 had no significant effect on CREB and mRNA, protein levels (total CREB and phospho-CREB). (B, C) Respective immunoblots are shown in the order of group presentation. Results are displayed as percentages of sed levels (represented by the 100% line). Each value represents the mean±S.E.M. (ANOVA, Fischer's test, * $P<0.05$).

Effect of exercise and D3 infusion on the signal transduction cascade p-CAMKII

Previously we found that the CAMKII signal transduction cascade is activated during exercise to modulate molecules mediating synaptic plasticity in the hippocampus (Vaynman et al., 2003). Therefore, we wanted to determine if energy metabolism has the ability to modulate the active form of CAMKII, i.e. p-CAMKII. Following, we infused D3 during exercise and measured the levels of p-CAMKII. Our results show that exercise significantly ($P<0.05$) increased the active form of the CAMKII cascade, p-CAMKII (144±7%) above sed levels (100±8%). D3 infusion abrogated the exercise-induced increase in p-CAMKII levels; thereby reducing them to sed levels (144±7% to 102±7%, exc vs. exc/D3). D3 application seemed to have no significant effect on p-CAMKII level in sedentary animals (100±8%, sed/D3; Fig. 7).

Exercise upregulates the mitochondrial uncoupler UCP2 in the hippocampus

To get a better indication of how exercise affects mitochondrial metabolism, we measured the mitochondrial uncoupler protein following 3, 7, and 28 days of exercise. We found that UCP2 expression increased signif-

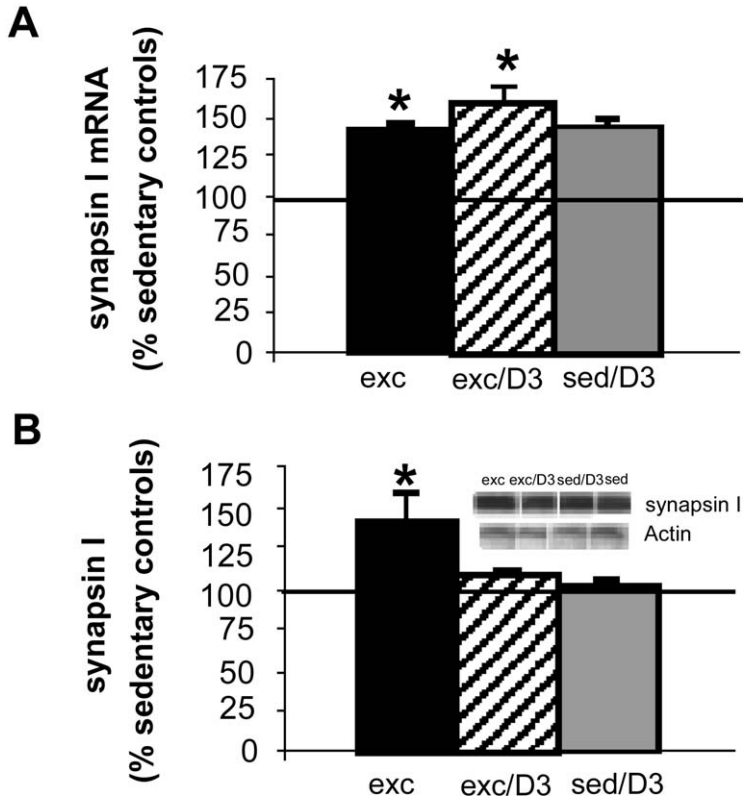

Fig. 6. D3 infusion modulates synapsin in the hippocampus. Exercise significantly increased synapsin I mRNA (A) and protein levels (B) above sed levels. (A) D3 infusion in sedentary animals increased synapsin I mRNA levels above sed levels. There was a significant effect for the joint application of D3 and exercise, such that synapsin I mRNA levels remained elevated above sed levels. (B) There was a significant effect of D3 with exercise, such that D3 infusion during exercise abrogated the exercise-induced increase in synapsin I protein levels. D3 had no significant effect on synapsin I protein levels. (B) Respective immunoblots are shown in the order of group presentation. Results are displayed as percentages of sed levels (represented by the 100% line). Each value represents the mean±S.E.M. (ANOVA, Fischer test, * $P<0.05$).

icantly ($P<0.05$) after only 3 days (123±11%) of exercise in the hippocampus and continued to remain so at 7 (131±7%) and 28 days (136±14%) of exercise (Fig. 8).

DISCUSSION

Cellular and molecular processes involved with the transmission of information across cells require energy supply

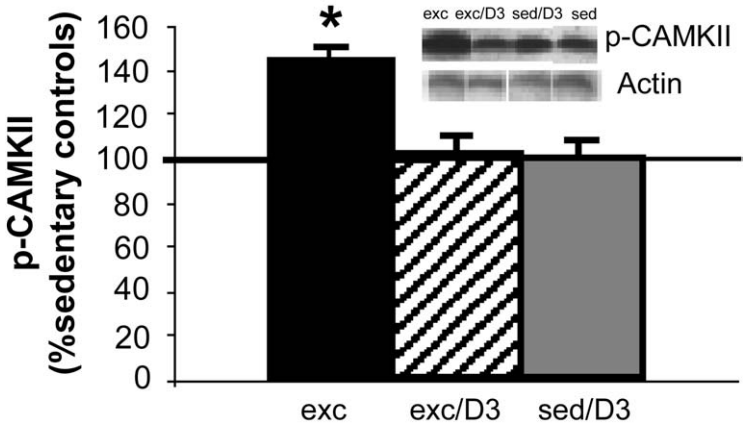

Fig. 7. D3 infusion modulates p-CAMKII in the hippocampus. Exercise significantly increased the protein levels of p-CAMKII above sed levels. Infusing D3 during exercise abrogated the exercise-induced increase in p-CAMKII levels, reducing them to the state of the sed group. Application of D3 had no significant effect on p-CAMKII levels in sedentary animals. Representative immunoblots corresponding to each group are shown overlaid on the actin controls for each group. Furthermost left immunoblot represents sed levels. Results are displayed as percentages of sed levels (represented by the 100% line). Each value represents the mean±S.E.M. (ANOVA, Fischer's test, * $P<0.05$).

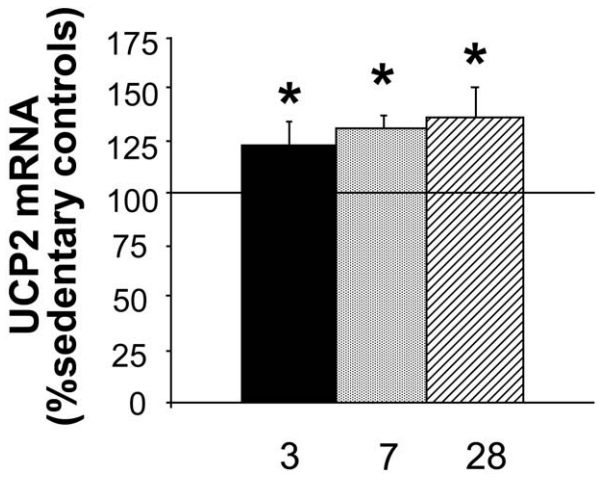

Fig. 8. Physical activity increases the expression of UCP2 in the hippocampus. Three days of exercise were sufficient to significantly increase the mRNA levels of UCP2 above sed levels. UCP2 mRNA levels remained significantly elevated after 7 and 28 days of exercise. Results are displayed as percentages of sed levels (represented by the 100% line). Each value represents the mean±S.E.M. (ANOVA, Fischer's test, * $P<0.05$).

(Attwell and Laughlin, 2001); accordingly the management of cellular energy is a crucial means by which to regulate synaptic function. However, the mechanisms involved with these events have remained obscure. Physical activity promotes changes in synaptic plasticity and cognitive function using the action of BDNF (Vaynman et al., 2004). The fact that BDNF plays a dual role in synaptic plasticity and energy metabolism provides the unique opportunity to evaluate how the energy demands imposed by behaviors, such as exercise, translate into modifications at neural circuits. Our results indicate that exercise modulates elements central to energy metabolism to subsequently affect synaptic plasticity. This is manifested as the "incorporation of new molecular species or the changes in the blend in the different molecules at the synapse," (Baudry et al., 1999), specifically an increase in key molecular components underlying learning and memory, i.e. BDNF, CREB, synapsin I, and p-CAMKII. These findings suggest a central mechanism by which physical activity and possibly other experiences use processes of energy metabolism to impact select aspects of hippocampal synaptic plasticity underlying cognitive function.

Exercise decreases OS: mechanisms to maintain homeostasis

Exercise intrinsically influences energy metabolism by involving mitochondrial oxidative phosphorylation. We found that exercise lowered cellular OS (Fig. 2A). OS seems to be related to energy mismanagement (Mattson and Liu, 2002). Therefore, it is possible that exercise may support BDNF-mediated synaptic plasticity by limiting OS to increase the efficiency of energy production from mitochondria. It is likely that physical activity achieves this capacity through the mitochondrion, as D3 acts to disrupt mitochondrial metabolism (Weitsman et al., 2003) by increasing free radical cytotoxicity to affect mitochondrial membrane potential (Weitsman et al., 2005). In fact, we found that D3 infusion prevented the ability of exercise to limit OS (Fig. 2A). This is consistent with the finding that D3 has a regulatory role over OS (Bondza-Kibangou et al., 2004). Additionally, exercise increased COX-II levels, an indicator of increased mitochondrial proliferation, and D3 infusion prevented the exercise-induced COX-II increase (Harper et al., 2002; Fig. 3). This finding may be related to the ability of exercise to limit hippocampal OS. Especially in the hippocampus, increases in mitochondrial number and ATP levels occur with decreases in the amount of OS (Diano et al., 2003). As exercise increases the mitochondrial uncoupling protein UCP2 at 3, 7, and 28 days following exercise (Fig. 8) and D3 has been found to inhibit UCP2 expression (Shi et al., 2002), it is possible that UCP2 contributes to the mechanism by which exercise uses the mitochondrion to limit OS and thereby to meet the energy demands imposed by exercise without the expense of high toxic OS (Arsenijevic et al., 2000; Negre-Salvayre et al., 1997; Andrews et al., 2005). UCP2 uncouples the hydrogen gradient from substrate phosphorylation (Sanchis et al., 1998), consequently reducing the amount of OS impacting the cell as a result of mitochondrial free radical production (Negre-Salvayre et al., 1997). Additionally, UCP2 can regulate mitochondrial proliferation (Dulloo and Samec, 2001; Andrews et al., 2005). Cells that have increased UCP2 expression have lower ATP production per mitochondrion but higher ATP production per cell (Harper et al., 2002; Garcia-Martinez et al., 2001; Horvath et al., 2003). Alternatively, as D3 infusion inhibited exercise-induced BDNF levels, D3 may act on BDNF through alternate mechanisms to UCP2, especially given that BDNF is involved in aspects of energy metabolism such as mitochondrial activity (El Idrissi and Trenker, 1999). Therefore, it is likely that the impact of exercise on synaptic-plasticity can be achieved by coordinating with mechanisms that regulate cellular energy metabolism. However, we cannot discard the possibility that D3 has other actions on energy metabolism. Importantly D3 has a postulated role in the modulation of obesity (Ye et al., 2001; Barger-Lux et al., 1995). Circulating D3 levels are elevated in obese humans (Andersen et al., 1988; Bell et al., 1985). D3 is a biologically active form of vitamin D that exerts a coordinated control over lipolysis and lipogenesis in adipocytes, such that increased D3 inhibits lipolysis and increases lipogenesis (Shi et al., 2001, 2002).

Exercise decreases OS, implications for the modulation of BDNF and CREB in the hippocampus

The ability of exercise to modulate OS may contribute to its capacity to affect BDNF-mediated plasticity. OS decreases the DNA-binding ability of CREB (Iwata et al., 1997), a prominent regulator of BDNF transcription (Tao et al., 1998), such that decreases in the DNA-binding ability of CREB are associated with decreases in BDNF expression (Vellucci et al., 2001). We found that infusing D3, a regulator of energy metabolism, directly into the hippocampus, modulates both BDNF and CREB. As BDNF regulates the transcription of CREB (Finkbeiner et al., 1997; Tully,

1997), OS-induced decreases in BDNF may contribute to a reduction in CREB expression. CREB is one of the best-described stimulus-induced transcriptional regulators with an evolutionary conserved role in long-term memory formation (Dash et al., 1990; Bourtchouladze et al., 1994; Tully et al., 1994; Yin et al., 1995; Yin and Tully, 1996; Frank and Greenberg, 1994). Exercise-enhanced learning and memory seem to be dependent on hippocampal BDNF

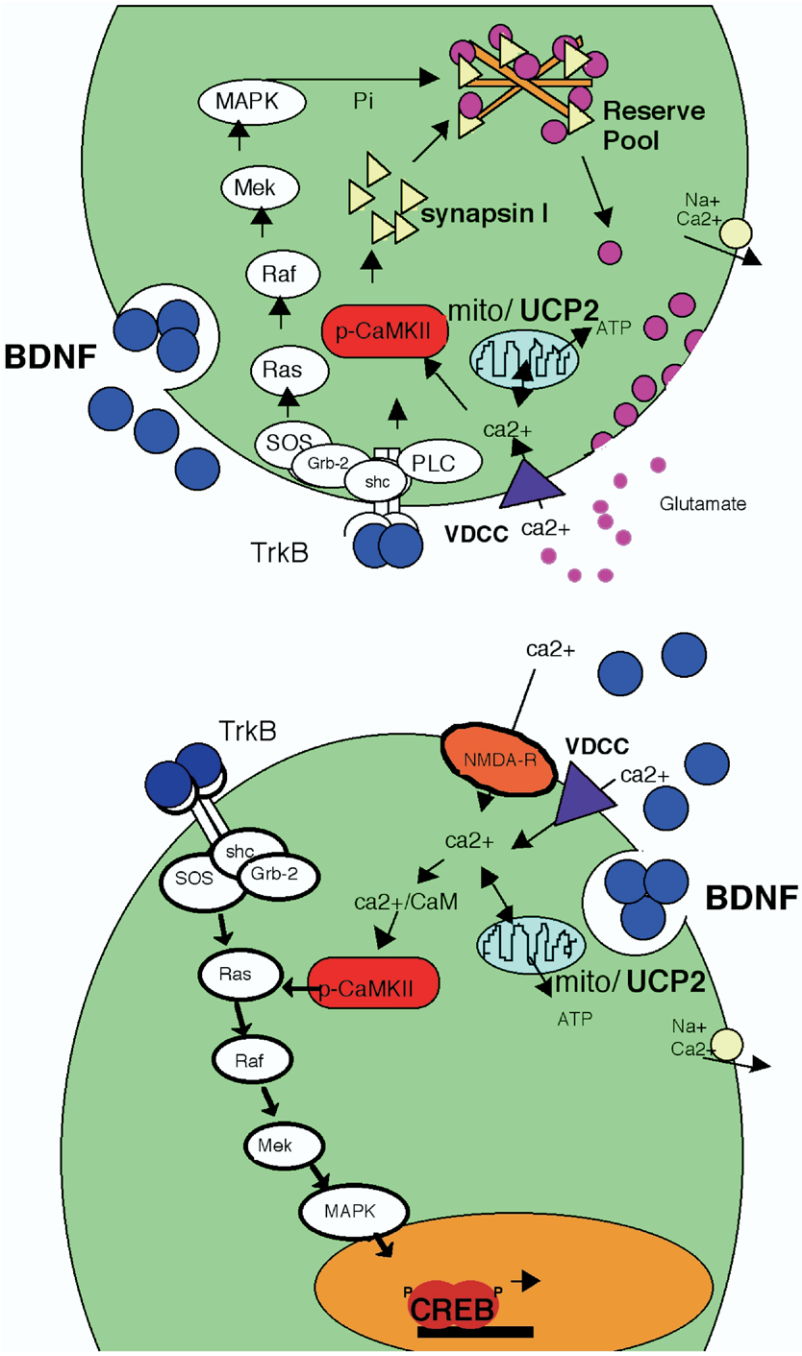

Fig. 9. Potential mechanism through which elements central to energy metabolism, mitochondria, and UCP2, may interact with the substrates for synaptic plasticity in the hippocampus under the action of exercise. BDNF molecules are depicted activating signal transduction cascades (MAP kinase and CAMKII) that activate CREB and synapsin I-mediated plasticity. UCP2, which contributes to calcium homeostasis, ATP production, and OS balance, is illustrated as one of possible mechanisms that can interact with these signal transduction cascades, especially CAMKII, to modulate the capacity of BDNF to regulate CREB and synapsin I during exercise. UCP2, situated in the mitochondria, is placed both near synaptic vesicles on the presynaptic membrane and near the NMDA-R on the postsynaptic membrane where it can serve as a calcium buffer during high calcium influxes. The presence of UCP2 at the edge of pre- and post- synaptic membranes may allow it to use its ability to modulate calcium, OS, and ATP production to influence vesicular release, by acting on vesicular release proteins such as synapsin I, and transcription, by impacting signal transduction cascades to activate transcriptional regulator such as CREB.

(Vaynman et al., 2004). Therefore, our current findings suggest that mechanisms central to hippocampal energy metabolism may play a role in modulating cognitive function. Additionally, studies have found that learning is associated with an increase in mitochondrial CREB and p-CREB levels (Bevilaqua et al., 1999).

The brain is an area whose high energy and oxygen consumption makes it susceptible to severe OS. It is believed that the accumulation of OS with age leads to the decline in neuroplasticity and the acceleration of neurodegenerative diseases (Beal, 1995). Not surprisingly, most neurodegenerative diseases share a cascade of molecular alterations that impinge on the balance of energy metabolism (Mattson 1997, 2000; Calabrese et al., 2001). Thus, as exercise uses elements of energy metabolism to modulate hippocampal synaptic plasticity, it may be a powerful tool for combating the neuro-cognitive decline associated with aging and neurodegenerative diseases.

Coupling energy metabolism to synaptic plasticity

The ability of energy metabolism to impact neurotransmission is supported by our finding that infusing D3 affects the ability of exercise to regulate synapsin I. Synapsin I is a presynaptic protein, involved in synaptic vesicle clustering and release (Jovanovic et al., 2000) and possibly learning (Garcia et al., 2004). Synapsin I is critical for establishing a synaptic vesicle reserve pool (Li et al., 1995; Takei et al., 1995), a requirement for preventing vesicular rundown during high frequency stimulation (Pieribone et al., 1995). Interestingly, exercise, as well as the dual application of exercise and inhibitor, significantly increased synapsin I mRNA levels above sed levels (Fig. 6A). In contrast, only exercise significantly increased synapsin I protein levels (Fig. 6B). As we have previously shown that BDNF regulates synapsin I expression during exercise (Vaynman et al., 2003), the likely effect of D3 on synapsin I mRNA levels is not due to a decrease in BDNF levels, as this event would advocate a concomitant decrement in synapsin I expression. However, the decrease in BDNF may have affected translational processes associated with the decrease in synapsin protein after D3 infusion. Given that OS regulates synapsin I levels in the hippocampus (Wu et al., 2004), our finding that D3 infusion moderates OS during exercise (Fig. 2), supports a basic mechanism by which behaviors may employ elements of energy metabolism to modulate components central to synaptic transmission.

Intersection of energy metabolism with BDNF signal transduction pathways

We found that infusing D3 directly into the hippocampus modulated the activated form of the CAMKII, i.e. p-CAMKII during exercise (Fig. 7). CAMKII is postulated to act as a molecular memory switch (Lisman et al., 2002), such that blocking CAMKII auto-phosphorylation results in memory deficits (Giese et al., 1998). We have previously found that exercise uses BDNF and the CAMKII cascade to promote changes in CREB and synapsin I expression in the hippocampus (Vaynman et al., 2003). CAMKII has been found to be localized to mitochondrial membranes (Liu and Jones, 1996) and its phosphorylation to promote its binding to the postsynaptic NMDA receptor (McNeill and Colbran, 1995; Meng et al., 2003; Leonard et al., 1999), a receptor that closely interacts with BDNF (Suen et al., 1997). As the mitochondria have privileged access to sequester NMDA-induced Ca^{2+} loads (Wang and Thayer, 2002), mitochondrial regulation may provide local Ca^{2+} to modulate the CAMKII pathway and possibly the convergent, BDNF-induced (Jovanovic et al., 2000) mitogen-activated protein kinase (MAPK) cascade (Platenik et al., 2000; Fig. 9). Access to CAMKII at the presynaptic membrane may also provide a mechanism by which mitochondrial function may modulate synapsin I levels, as CAMKII also localizes with presynaptic vesicles at the synapse and functions as a binding protein for synapsin I (Benfenati et al., 1992, 1996).

CONCLUSIONS

The benevolent actions of exercise on brain health are well known. However, the mechanisms involved remain obscure. The longstanding question has been how changes in energy metabolism imposed by exercise and other experiences can influence the plasticity of neural circuits. Our results show that exercise modulates molecular systems involved with mechanisms that process energy. Changes in energy metabolism activated by exercise appear to be significant for the modulation of BDNF-mediated synaptic plasticity in the hippocampus. It is likely that these events extend to comprise basic mechanisms supporting homeostatic functions in the CNS that may ultimately serve mental health. It has been shown that cognitive enhancement associated with exercise is related to elevated BDNF. Accordingly, it remains to be shown how the modulation of BDNF by energy metabolism during exercise can influence specific aspects of cognitive function.

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