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Hippocampal BDNF mediates the efficacy of exercise on synaptic plasticity and cognition

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Abstract

We found that a short exercise period enhanced cognitive function on the Morris water maze (MWM), such that exercised animals were significantly better than sedentary controls at learning and recalling the location of the platform. The finding that exercise increased brain-derived neurotrophic factor (BDNF), a molecule important for synaptic plasticity and learning and memory, impelled us to examine whether a BDNF-mediated mechanism subserves the capacity of exercise to improve hippocampal-dependent learning. A specific immunoadhesin chimera (TrkB-IgG), that mimics the BDNF receptor, TrkB, to selectively bind BDNF molecules, was used to block BDNF in the hippocampus during a 1-week voluntary exercise period. After this, a 2-trial-per-day MWM was performed for 5 consecutive days, succeeded by a probe trial 2 days later. By inhibiting BDNF action we blocked the benefit of exercise on cognitive function, such that the learning and recall abilities of exercising animals receiving the BDNF blocker were reduced to sedentary control levels. Inhibiting BDNF action also blocked the effect of exercise on downstream systems regulated by BDNF and important for synaptic plasticity, cAMP response-element-binding protein (CREB) and synapsin I. Specific to exercise, we found an association between CREB and BDNF expression and cognitive function, such that animals who were the fastest learners and had the best recall showed the highest expression of BDNF and associated CREB mRNA levels. These findings suggest a functional role for CREB under the control of BDNF in mediating the exercise-induced enhancement in learning and memory. Our results indicate that synapsin I might also contribute to this BDNF-mediated mechanism.

Introduction

Over the past two decades, both animal and human studies have shown the capacity of exercise to benefit cognitive function. Notably, exercise enhances memory and cognition (Fordyce & Wehner, 1993; Kramer *et al*., 1999), facilitates functional recovery following brain injury (Grealy *et al*., 1999), and counteracts the mental decline associated with ageing (Laurin *et al*., 2001). A characteristic finding is the exercise-induced increase in brain-derived neurotrophic factor (BDNF) in the hippocampus, an area involved in learning and memory formation (Neeper *et al*., 1997; Vaynman *et al*., 2003). Given the importance of BDNF for synaptic plasticity and learning and memory, it has been proposed that these exercise-induced increases in hippocampal BDNF levels might underlie the ability of exercise to enhance cognitive function. Nevertheless, the central question remains whether the exercise-induced enhancement in learning and memory is dependent upon the action of hippocampal BDNF during exercise.

BDNF is a member of the neurotrophin family known to play a prominent role in the survival, growth, and maintenance of neurons during development (Leibrock *et al*., 1989; Barde, 1994) and more recently, the ability to modulate synaptic-plasticity in the adult brain (Lo, 1995). This capability of BDNF to impact synaptic plasticity can be seen in the diverse tasks it manages; BDNF regulates axonal and dendritic branching and remodelling (Shimada *et al*., 1998; Lom & Cohen-Cory, 1999; McAllister *et al*., 1999; Yacoubian & Lo, 2000), synaptogenesis in arborizing axon terminals (Alsina *et al*., 2001), the efficacy of synaptic transmission (Lohof *et al*., 1993; Kang & Schuman, 1995; Boulanger & Poo, 1999; Kafitz *et al*., 1999), and the functional maturation of excitatory and inhibitory synapses (Seil & Drake-Baumann, 2000; Rutherford *et al*., 1998; Vicario-Abejon *et al*., 1998). BDNF gene deletion or inhibition (Figurov *et al*., 1996; Kang *et al*., 1997) produces a deficit in long-term potentiation (LTP), the transcription-dependent electrophysiological correlate of learning and memory (Nguyen & Kandel, 1996). Moreover, this deficit in synaptic function can be amended by the exogenous application (Patterson *et al*., 1996) or overexpression (Korte *et al*., 1995) of BDNF.

Previously, we found that exercise induces synaptic plasticity markers in the hippocampus through a BDNF-mediated mechanism, particularly increasing the mRNA levels of calcium and cAMP response-element-binding (CREB) protein and synapsin I (Gomez-Pinilla *et al*., 2001; Vaynman *et al*., 2003). CREB is one of the best described stimulus-induced transcriptional regulators, notable for its involvement in adaptive responses (Finkbeiner *et al*., 1997; Finkbeiner, 2000), but particularly famous for its evolutionary conserved role in long-term memory (LTM) formation (Dash *et al*., 1990; Bourtchouladze *et al*., 1994; Frank & Greenberg, 1994; Tully *et al*., 1994; Yin *et al*., 1995; Abel & Kandel, 1998). Synapsin I is a presynaptic phosphoprotein, which has been shown to modulate transmitter release (Jovanovic *et al*., 2000), the formation and maintenance of the presynaptic structure (Melloni *et al*., 1994; Takei *et al*., 1995) and axonal elongation (Akagi *et al*., 1996).

Although previous work has shed some light on underlying synaptic plasticity changes the brain undergoes with exercise, the mechanisms

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responsible for bestowing the exercise-induced enhancement in cognitive function are as yet poorly understood. Therefore, in this current study we investigated the causal link between exercise-induced cognitive improvement and BDNF action. To block the action of BDNF, we employed a specific immunoadhesin chimera (TrkB-IgG) that mimics the BDNF receptor, TrkB, to selectively bind BDNF molecules. We injected TrkB-IgGs into the hippocampus before the start of wheel running, using a microbead vehicle that enables the inhibitory effect of the BDNF blocker to last throughout the 1-week exercise period, and measured Morris water maze (MWM) performance and mRNA levels of BDNF, TrkB, synapsin I and CREB. It is of significant clinical interest to determine if exercise utilizes BDNF action to exert its beneficial effects on neuronal and cognitive plasticity, as exercise may be potentially combined with other interventions which might impact BDNF-mediated mechanisms.

Materials and methods

Exercise paradigm

Adult male Sprague-Dawley (Charles River) rats (3 months old) were randomly assigned into four groups: sedentary with cytochrome C (cytC) injection (sed/cytC; $n = 7$); exercise with cytC injection (exc/cytC; $n = 7$); sedentary with TrkB-IgG injection (sed/TrkB-IgG; $n = 7$); and exercise with TrkB-IgG injection (exc/TrkB-IgG; $n = 7$). All rats were housed individually in standard polyethylene cages in a 12/12 h light/dark cycle at 22–24 °C, with food and water available *ad libitum*. We chose a voluntary exercise paradigm because it simulates aspects of human behaviour in which animals choose how much to run. 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). All groups were allowed to acclimate to their respective environments for 1 week before the start of experiments. Animals were exercised for a period of 1 week before MWM training, during which respective drug treatments were given to groups. The choice of a short 1-week exercise period was based upon the findings that 4 days of exercise are sufficient to enhance performance on the MWM task (Shaw *et al*., 2003) and increase the expression of synaptic plasticity markers believed to underlie mechanisms supporting cognitive function (Molteni *et al*., 2002; Vaynman *et al*., 2003). As in Shaw *et al*. (2003), in order to reproduce the effects of a short exercise period on cognitive function, we required that each rat ran a minimum of 100 m each night. The control rats were confined to a cage with no access to a running wheel. Animals continued in their respective experimental conditions for the duration of the experiment. All animals were killed by decapitation the morning following the last treatment day and their hippocampi were rapidly dissected out, immediately placed on dry ice, and stored at −70 °C. These studies were performed in accordance with the United State National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by UCLA Animal Research Committees.

Drugs

Recombinant human TrkB-IgG chimera was acquired from R & D System, Inc. (Minneapolis, MN, USA) in powder form. This chimerical protein comprises the intracellular domains of human TrkB and the Fc domain of IgG, and has been shown to be a highly potent and specific antagonist of BDNF action (Shelton *et al*., 1995). We used cytochrome C (cytC), obtained in powder from Sigma (St. Louis, MO, USA) as the control drug as it has been used as a standard control for microbead injections (Lom & Cohen-Cory, 1999; Vaynman *et al*., 2003). CytC was dissolved in sterile distilled water, with stock concentration of 100 ng/µL. Fluorescent latex microbeads, used as the vehicle for drug insertion into the hippocampus, were purchased from Lumaflour Corp., Naple, FL. The sterile phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) was added to the vial to prepare a stock solution (100 µg/mL).

Preparation of microbeads

Infusion of TrkB-IgGs 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). We prepared microbeads (Lumaflour, Naples, FL, USA) by the methods described previously (Riddle *et al*., 1997; Lom & 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 TrkB-IgG (5 µg/µL in PBS with BSA, Croll *et al*., 1998) and cytC (100 ng/µL in sterile water, Lom & 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. These two drugs were administered via injection of fluorescent latex microbeads directly into the right hippocampus, resulting in a consistent and effective blockade of targets as shown in the results. In a previous publication, we have shown that microbead inhibition was effective for 3 days (Vaynman *et al*., 2003). In a new set of studies, in which we used the same protocol, we have found that microbead inhibition lasts up to 7 days (our unpublished observations). Previous studies by Quattrochi *et al*., (1989), Riddle *et al*. (1995, 1997) and Lom & Cohen-Cory (1999) have reported successful delivery by microbeads of bioactive agents such as neurotrophins and neurotransmitter agonists/antagonists into highly localized brain regions.

Injection of drugs into the hippocampus

Exercise and sedentary rats received TrkB-IgG or the standard control cytC injection. We used a unilateral injection to the right hippocampus to be consistent with previous blocking experiments (Vaynman *et al*., 2003). We did not use the contralateral hippocampus as a control because a unilateral injection can cause changes on the contralateral side due to connecting fibres (Amaral & Witter, 1989). Injections were given to all animals once before the onset of the running period, and administered early in the morning such that an ample recovery time permitted all animals to begin running that same evening. All animals were anaesthetized by isoflurane (2–2.5%) utilizing the Mobile Laboratory Animal Anaesthesia System, and positioned in a stereotaxic apparatus that was used to secure the animal and to measure the sight for injection. TrkB-IgG or cytC 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. 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 and only those showing characteristic markings of microbeads of the right hippocampus were used for mRNA measurements. The microbead injection site was additionally verified by florescence microscopy (shown in Fig. 1), using an Olympus BX51 microscope.

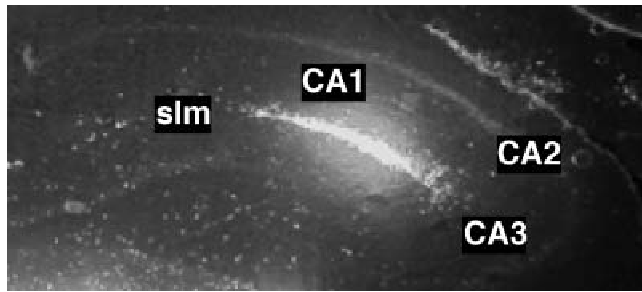

FIG. 1. Tissue section in the sagittal plane of the right hippocampus, showing the microbead injection site concentrated in the stratum lacunosum moleculare (slm) using florescence microscopy. Hippocampal areas have been labelled for convenience; CA1, CA2 and CA3.

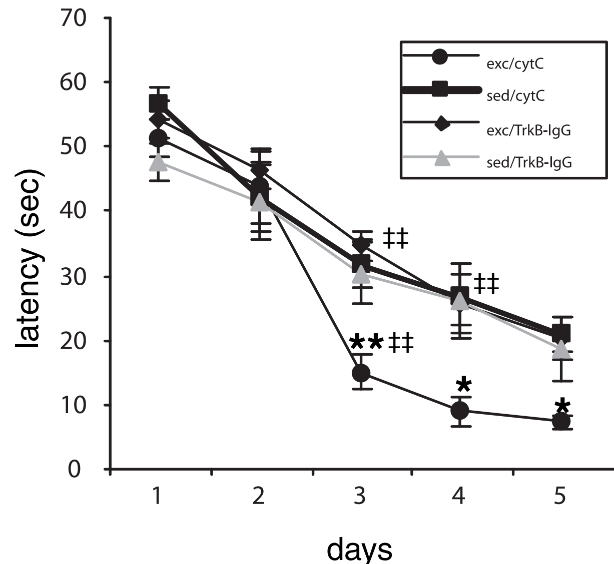

FIG. 2. Effect of blocking BDNF action during the exercise period on learning using the MWM task. Exercise effectively improved learning ability, as exercised animals took significantly less time to find the platform (shorter escape latencies in the exc/cytC group). Blocking BDNF action during exercise abolished the exercise-induced enhancement of learning ability, as exercised animals given the BDNF blocker took longer to find the platform than exercised control animals (exc/TrkB-IgG vs. exc/cytC). In fact, blocking BDNF action in exercised animals resulted in escape latency comparable sedentary control animal (exc/TrkB-IgG vs. sed/cytC). ‡‡Exercised animals (exc/cytC and exc/TrkB-IgG rats) began to show a significant improvement in learning acquisition on day 3 of MWM training (as compared to days 1and 2). In contrast, sedentary animals (sed/cytC and sed/TrkB-IgG rats) showed a significant improvement in learning acquisition on day 4 of MWM training (as compared to days 1 and 2). Data are expressed as mean ± S.E.M. (ANOVA, Fischer test, Scheffe F-test, *$P < 0.05$, **,‡‡$P < 0.01$; *represents comparison between groups, ‡‡represents comparison within groups).

Cognitive testing

To evaluate the effect of exercise and TrkB-IgG inhibition of BDNF activity on memory functions, all rats were tested, using the Morris water maze, for spatial memory acquisition and retention (Morris *et al*., 1982; Sutherland *et al*., 1982). The swimming pool (130 cm diameter, 50 cm height) was divided into four quadrants. As previously discussed in Molteni *et al*. (2002), the quadrant housing the escape platform (12 cm diameter), was fixed in a permanent position 2 cm under the water surface during the course of the MWM training procedure and was defined as the target zone. The water, kept at a steady 22 ± 2 °C, was made opaque with white nontoxic biodegradable dye to prevent the rats from seeing the platform. The rats were trained in the MWM for 5 consecutive days using the challenging 2-trial-per-day regimen previously found to discern learning difference in exercise and sedentary animals (van Praag *et al*., 1999). We changed the 6 day training paradigm to a 5 day training paradigm which was more conducive to assessing the shorter exercise period we employed (Shaw *et al*., 2003). Overall, the 2-trial-per-day, 5 day MWM training paradigm was chosen for optimal discernment of learning differences in animals exposed to a short-term exercise regimen. The animals were placed into the tank facing the wall from one of the equally spaced start locations, which were randomly altered every trial. Spatial reference cues around the pool were maintained constant through out the duration of the MWM training and probe trials. Each trial lasted until the rat found the platform or for a maximum duration of 60 s. If the rat failed to find the platform, it was gently placed on the platform. At the end of each trial, the rat was allowed to rest on the platform for 10 s. The time to reach the platform (escape latency) was recorded for each animal. To assess spatial memory retention, a probe trial was performed 2 days after the last training trial, during which the platform was removed from the pool, while all other factors remained constant. As previously described (Molteni *et al*., 2002, 2004), rats were allowed to swim for 60 s, during which the percentage of time spent in each quadrant was calculated and their swim paths were semiautomatically recorded by a video tracking system (Smart: Spontaneous Motor Activity Recording and Tracking. #35E4F-FA9, Pan Laboratory s.l). The location of the platform was designated as quadrant P (Fig. 2B).

Isolation of total RNA and real-time quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated using RNA STAT-60 kit (TEL-TEST, Inc., Friendswood, TX, USA) as per the manufacturer's protocol. Quantification was carried out by absorption at 260 nm. The mRNAs for BDNF, TrkB, CREB and synapsin I were measured by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) using a 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-labelled 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 (Griesbach *et al*., 2002; Molteni *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:

BDNF
(5′-AGTCATTTGCGCACAACTTTAAAAGTCTGCATT-3′);
forward (5′-GGACATATCCATGACCAGAAAGAAA-3′);
reverse (5′-GCAACAAACCACAACATTATCGAG-3′);

TrkB
(5′-TGC ACGTCTGGCCGCTCCTAACC-3′);
forward (5′-CCCAATTGTGGTCTGCCG-3′);
reverse (5′-CTTCCCTTCCTCCACCGTG-3′);

CREB
(5′-CATGGCACGTAATGGAGACTACCGCA-3′);
forward (5′-CCGCCAGCATGCCTTC-3′);
reverse (5′-TGCAGCCCAATGACCAAA-3′);

synapsin I
(5′-CATGGCACGTAATGGAGACTACCGCA-3′);
forward (5′-CCGCCAGCATGCCTTC-3′);
reverse (5′-TGCAGCCCAATG ACCAAA-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′-CCGACTCTTGCCCTTC GAAC-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.

Western blot

To get a better measure of the active sate of CREB, the expression of p-CREB was assessed by immunoblotting. Hippocampal tissue was homogenized in a lysis buffer containing 137 mM NaCl, 20 mM Tris-HCl pH 8.0, 1% NP40, 10% glycerol, 1 mM PMSF, 10 μg/mL aprotinin, 0.1 mM benzethonium chloride and 0.5 mM sodium vanadate. The homogenates were then centrifuged, the supernatants were collected and total protein concentration was determined according to the Micro BCA procedure (Pierce, Rockford, IL, USA), using BSA as standard. Equal amounts (25 μg) of protein samples were separated by electrophoresis on a 10% polyacrilamide gel and electrotransferred to a nitrocellulose membrane. The filters were blocked with 5% nonfat milk. Membranes were then incubated with anti-actin antibody (1 : 2000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-p-CREB antibody (1 : 1000, Upstate, Lake Placid, NY, USA), followed by anti-rabbit IgG horseradish peroxidase-conjugate. After rinsing with buffer, the 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 quantified using NIH Image software, normalized for actin level.

Statistical analyses

We used GAPDH for RT-PCR as an internal standard as described previously in our other studies (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 to 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 BDNF, TrkB, CREB, and synapsin I were corrected by subtracting values for GAPDH as described previously (Greisbach *et al*., 2002; Molteni *et al*., 2002). These corrected values were used to make cross group comparisons. The mean values for the mRNA levels were computed for all four groups following completion of exercise and MWM training and compared using analysis of variance (ANOVA). The analysis of maze data was performed in accordance with techniques described in Molteni *et al*. (2002), using an ANOVA with repeated measures. The mean values of distances (Km) run over the exercise period were computed for the exercise groups and compared using *t*-test. A Fischer-test was used for cross group comparisons. A Scheffe *F*-test was used to compare learning differences between days for each group. Results were expressed as the mean percent of control values for graphic clarity and represent the mean ± SEM. We used a regression analysis to evaluate the association between mRNA expression for all the combinations of BDNF, TrkB, CREB and synapsin I, for each of the four groups. Additionally, we used regression analysis to evaluate the association between mRNA expression and MWM performance, both the acquisition (escape latency slope) and the consolidation phase (percentage time spent in P quadrant during probe trial), in each group.

Results

Microbead injection site

We inspected the microbead injection location via fluorescence imaging. As shown in Fig. 1, the microbead carrier is centralized to the injection site, in the stratum lacunosum moleculare (slm) of the hippocampus, while the drug transported is free to disperse to other areas of the hippocampus. Our microbead infusion site is consistent with our previous study, which used drug blockers coupled to microbeads (Vaynman *et al*., 2003).

MWM performance: learning acquisition

To assess spatial learning acquisition, we used a challenging 2-trial-per-day paradigm, which has proven to be sufficiently sensitive for discerning learning differences between exercise and sedentary animals (van Praag *et al*., 1999). Animals were trained every day for 5 consecutive days on the MWM task. Results showed that the escape latencies were similar between all four groups on days 1 and 2 of MWM training (Fig. 2). We found a significant difference in latency between the exc/cytC group and all the other three groups (sed/cytC, exc/TrkB-IgG, and sed/TrkB-IgG; Fig. 2). We found that exc/cytC rats had significantly shorter escape latencies on day 3 (14.57 ± 2.71 s, $P < 0.01$), day 4 (8.5 ± 2.47 s, $P < 0.05$), and day 5 (6.93 ± 0.99 s, $P < 0.05$) of MWM training than sed/cytC control rats (31.58 ± 3.63 s, 26.71 ± 4.74 s, and 20.5 ± 2.78 s, respectively; Fig. 2). Injection of TrkB-IgGs into the hippocampus significantly abrogated the effect of exercise on MWM training performance, reducing the escape latency of exc/TrkB-IgG rats on days 3 (34.2 ± 2.39 s, $P < 0.01$), 4 (25.25 ± 4.38 s, $P < 0.05$), and 5 (19.94 ± 3.18 s, $P < 0.05$) to sed/cytC control level (Fig. 2). In contrast, TrkB-IgG injection did not have any significant effect on sedentary rats; the sed/IgG group had similar escape latencies on days 3 (30 ± 4.92 s), 4 (25.64 ± 5.92 s), and 5 (18.21 ± 5.05 s) to those of sed/cytC controls (Fig. 2).

The performance of groups during the acquisition phase can be better appreciated by looking at the slope of the escape latencies between groups. The exc/cytC group had a significantly ($P < 0.05$) steeper escape latency slope (m =12.29) than the sed/cytC control group (m = −8.66), illustrating that the exc/cytC rats had acquired the MWM task faster than sed/cytC control rats. Inhibiting BDNF action with TrkB-IgGs eliminated the increased rate of learning acquisition produced by exercise, as exc/TrkB-IgG (m = −8.88) and sed/cytC control animals had a similar slope of acquisition. TrkB-IgG seemed to have no effect on MWM acquisition during sedentary conditions as there was no significant difference in the slopes of sed/IgG animals (m = −7.33) and sed/cytC controls.

To determine when a significant enhancement in MWM acquisition occurred, we performed an ANOVA between days for each

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group. We found that escape latency in the exc/cytC control group was significantly reduced on day 3 of MWM training (as compared with days 1 and 2), indicating that they had a significant improvement in learning acquisition on day 3 of MWM training (‡‡$P < 0.01$, Fig. 2). In contrast, both sedentary groups, i.e. sed/cytC and sed/TrkB-IgG, did not show a significant reduction in escape latency until day 4 (as compared with days 1 and 2, **$P < 0.01$; Fig. 2). We found that the exc/TrkB-IgG group retained the significant within group improvement on day 3 (‡‡$P < 0.01$; Fig. 2), but that the performance of the exc/cytC group was also significantly better than exc/TrkB-IgG rats on day 3 (between groups, $P < 0.01$; Fig. 2).

MWM performance: memory retention

To evaluate memory retention, we performed a probe trial 2 days after the last MWM training day. Rats were allowed to swim for 60 s in the pool in which they received their training, but with the escape platform removed. The percentage of time spent in the probe quadrant, which previously housed the platform (quadrant P), was calculated for each animal. We found that the exc/cytC group showed a clear preference for the P quadrant over sed/cytC rats, as they spent a significantly ($P < 0.05$) greater percentage of time in quadrant P (48.27 ± 3.14%) than sed/cytC controls (33.95 ± 4.64%). Administration of TrkB-IgG fully prevented the exercise-induced preference for the target quadrant, such that there was no difference between the amount of time spent by exc/TrkB-IgG rats (32.13 ± 3.22%) and sed/cytC controls in quadrant P (Fig. 3A). TrkB-IgG seemed not to affect the preference of sed/TrkB-IgG rats for quadrant P, as there was no significant difference in the percentage of time spent in quadrant P between sed/TrkB-IgG (31.63 ± 3.26%) and sedentary control rats (Fig. 3A).

We found that the results of our study showed an effect of blocking BDNF action during exercise, as sedentary groups, sed/cytC and sed/TrkB-IgG, did not show any significant differences in their escape latencies and preference for the target zone (quadrant P) in the MWM task, as would be achieved if BDNF was blocked during the actual MWM task (Mu *et al*., 1999). This effect was reproduced and very apparent in our mRNA results, discussed in the subsequent sections. We recorded the running distances for each exercise group and found that the average distance (Km) run over the 1-week period did not differ significantly between the exercise groups, exc/cytC (6.83 ± 1.91) and exc/TrkB-IgG (7.30 ± 2.11).

Effect of exercise and BDNF blockage on the mRNA levels of BDNF and its TrkB receptor

BDNF uses TrkB as the primary receptor to transduce activity into signal transduction cascades (Barbacid, 1994). Accordingly, we quantified hippocampal TrkB as well as BDNF mRNA levels in exercised animals. We found that exercise significantly ($P < 0.05$) increased the mRNA levels of BDNF (130 ± 13%) and its TrkB receptor (120 ± 6%) in the hippocampus above sed/cytC controls

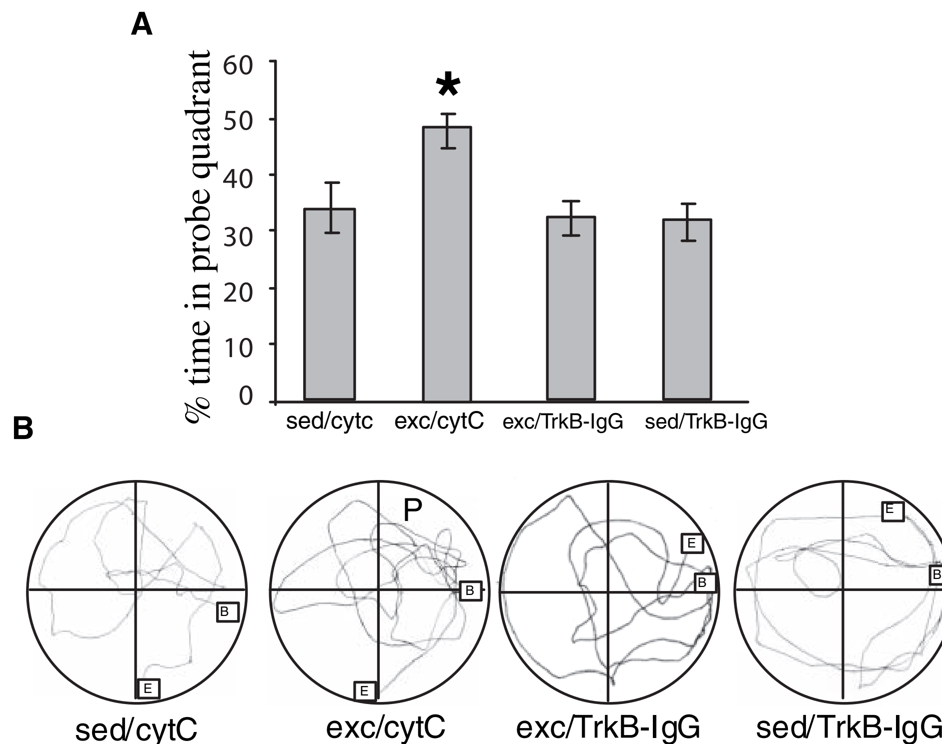

FIG. 3. Effect of blocking BDNF action during the exercise period on memory retention using the probe trial on the MWM task. (A) Exercise increased the memory retention on the MWM task as indicated by the finding that exercised animals spent significantly more time in quadrant P than sedentary controls (exc/cytC vs. sed/cytC). Blocking BDNF action during exercise abolished this exercise-induced preference for the P quadrant (exc/TrkB-IgG vs. exc/cytC), such that exercised animals receiving the BDNF blocker spent as much time in the P quadrant as sedentary control animals (exc/TrkB-IgG vs. sed/cytC). Blocking BDNF action did not have an effect on the preference of sedentary animals for the P quadrant (sed/TrkB-IgG vs. sed/cytC). (B) Representative samples of trials traveled during the probe test (B, begin, E, end, *P*, quadrant which previously housed the platform), illustrating the marked preference of the exc/ctyC group for the P quadrant as compared to all other groups. Each value represents the mean ± SEM (ANOVA, Fischer test, *$P < 0.05$).

(Fig. 4A and B: exc/cytC group). Inhibiting the action of BDNF with TrkB-IgGs fully blocked the exercise-induced effect on the mRNA levels of BDNF (130 ± 13% to 99 ± 5%) and its TrkB receptor (120 ± 6% to 106 ± 10%), effectively reducing them to the BDNF and TrkB mRNA levels of sed/cytC controls (Fig. 4A and B: exc/TrkB-IgG vs. sed/cytC). We found that blocking BDNF action with TrkB-IgGs did not have an effect on BDNF and TrkB mRNA levels in the sedentary condition, such that there was no significant difference between the sed/TrkB-IgG and the sed/cytC groups (Fig. 4A and B).

Effect of exercise and BDNF blockage on plasticity markers: CREB and synapsin I mRNA levels

Exercise significantly ($P < 0.05$) increased the mRNA levels of CREB (115 ± 4%) and synapsin I (129 ± 8%) in the exc/cytC groups above those of sed/cytC controls (Fig. 4C and D). Blocking BDNF action with TrkB-IgGs was sufficient to completely abrogate the exercise-induced increase in synapsin I (129 ± 8% to 86 ± 6%) and CREB (115 ± 4% to 92 ± 3%) mRNA levels in exc/TrkB-IgG rats, thereby effectively reducing them to sed/cytC control levels (Fig. 4C and D). Blocking the action of BDNF with TrkB-IgGs seemed to have no effect during the

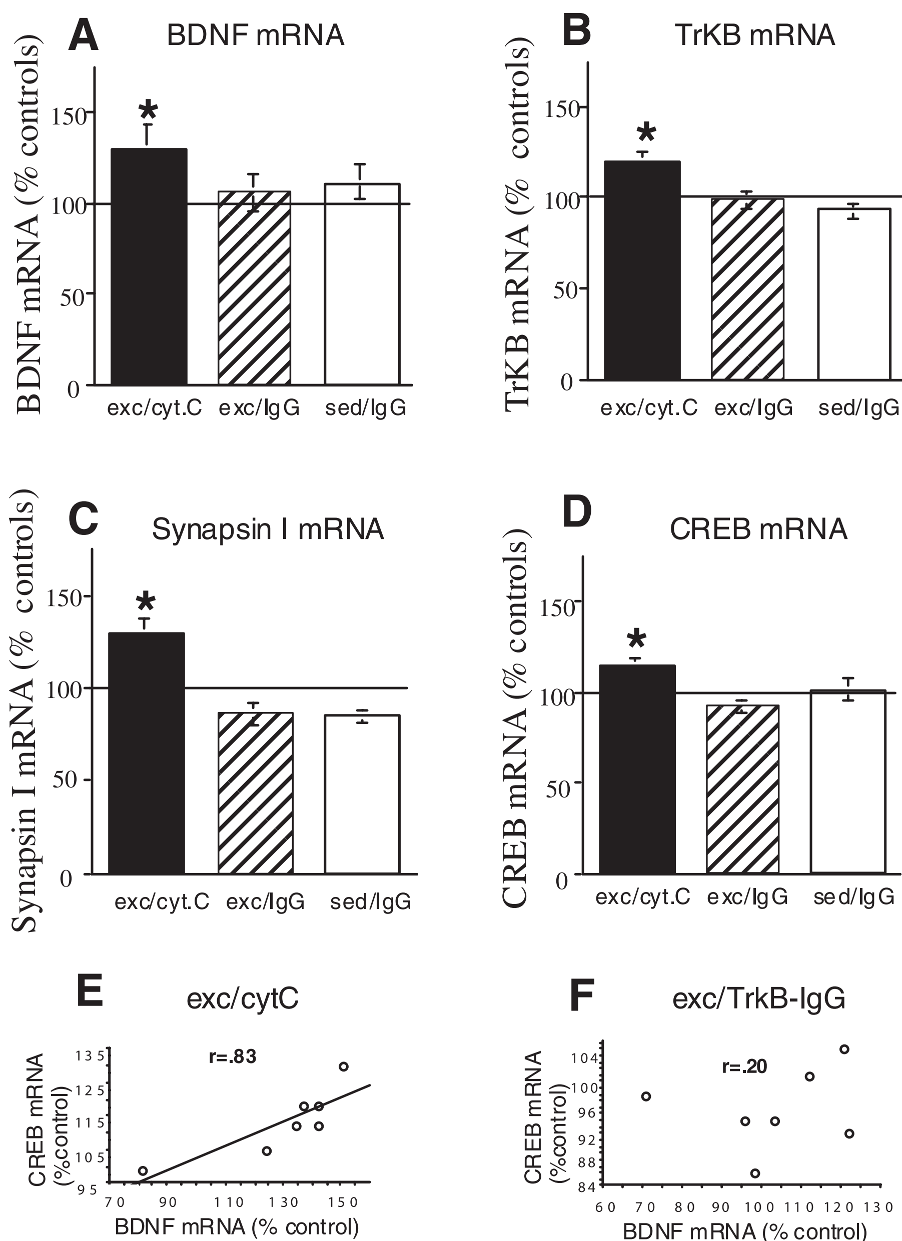

FIG. 4. Effect of blocking BDNF action during the exercise period on the mRNA levels of BDNF, TrkB, synapsin I, and CREB, as measured after the probe trial. Results are displayed as percentages of sed/cytC control levels (represented by the 100% line). Exercise significantly increased the mRNA levels of BDNF (A), TrkB (B), synapsin I (C) and CREB (D) above those of sedentary controls. Blocking BDNF action significantly reduced the expression of BDNF (A), TrkB (B), synapsin I (C), and CREB (D) to sedentary control levels. Blocking BDNF action had no significant effect on the expression of these markers in sedentary animals. E-F show the exercise dependent association between CREB and BDNF mRNA levels. (E) A significant positive correlation was found between hippocampal BDNF and CREB mRNA levels during exercise ($r = 0.83$. $P < 0.05$) which was disrupted in F by blocking BDNF in the exc/TrkB-IgG group ($r = 0.20$). The association is illustrated by the computer generated best-fit line. Each value represents the mean ± S.E.M. (ANOVA, Fischer test, $^*P < 0.05$).

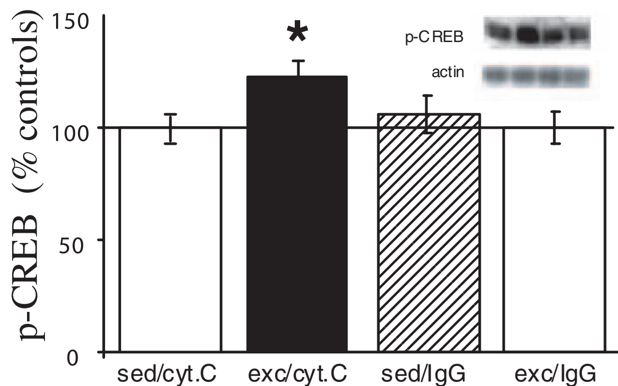

FIG. 5. Effects of exercise and blocking BDNF action during the exercise period on the protein levels of pCREB, as measured after the probe trial by Western blot. Exercise significantly increased pCREB levels above those of sedentary controls (exc/cytC vs. sed/cytC rats). Blocking BDNF action significantly reduced pCREB back to sedentary control levels (exc/TrkB-IgG vs. sed/cytC). Blocking BDNF action had no significant effect on the expression of pCREB in sedentary animals (sed/TrkB-IgG vs. sed/cytC). Respective immunoblots are shown in the order of group representation. Each value represents the mean ± SEM (ANOVA, Fischer test, $^*P < 0.05$).

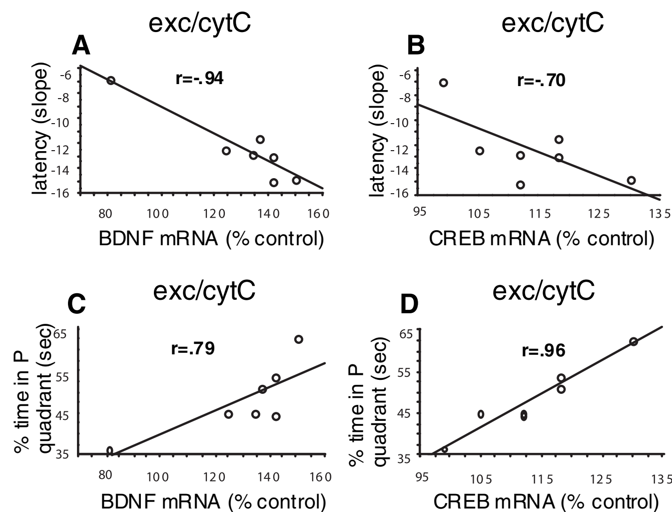

FIG. 6. Exercise dependent association between BDNF and CREB mRNA with cognitive function, as measured by learning acquisition and retention on the MWM task. A significant negative association between the slope of escape latencies and (A) BDNF ($r = -0.94$, $P < 0.05$) and (B) CREB ($r = -0.70$, $P < 0.05$) mRNA levels. A significant positive correlation between the percentage of time spent in the platform quadrant P and (C) BDNF ($r = 0.79$, $P < 0.05$) and (D) CREB ($r = 0.96$, $P < 0.05$) mRNA levels. The association is illustrated by the computer-generated best-fit line.

sedentary condition, as synapsin I (85 ± 3%) and BDNF (112 ± 9%) mRNA levels in the sed/TrkB-IgG group did not significantly differ from those of sed/cytC controls (Fig. 4C and D). We ran a regression analysis between mRNA levels in individual animals. We found that, in exc/cytC animals, BDNF mRNA levels correlated significantly with CREB mRNA levels ($r = 0.83$, $P < 0.05$), which did not exist in any of the sedentary groups. We found that TrkB-IgG infusion disrupted this correlation ($r = 0.20$; Fig. 4E and F, respectively).

As CREB has been shown to be highly critical for memory (Dash *et al*., 1990; Bourtchouladze *et al*., 1994; Yin *et al*., 1995), we measured the phosphorylated form of CREB (p-CREB), to get a better indication of its active state. We found that exercise significantly ($P < 0.05$) increased p-CREB levels (123 ± 7%) in the exc/cytC group above sed/cytC controls (Fig. 5). Inhibiting BDNF action in exc/TrkB-IgG animals fully blocked the exercised-induced increase in p-CREB levels (123 ± 7% to 106 ± 7%). Blocking BDNF action with TrkB-IgGs did not decrease p-CREB levels in the sedentary condition, and such p-CREB levels of the sed/TrkB-IgG rats (100 ± 8%) were not significantly different from those of sed/cytC controls (100 ± 5%; Fig. 5).

Behavioural and molecular interaction during exercise

In exc/cytC rats, we found that the slope of escape latencies was significantly and negatively associated with hippocampal BDNF ($r = -.94$, $P < 0.05$) and CREB ($r = 0.70$, $P < 0.05$) expression (Fig. 6A and B, respectively): There was a tendency for the best spatial learners to have the highest hippocampal BDNF and CREB mRNA levels. Moreover, exc/cytC rats expressed a significant positive correlation between performance on the probe trial (% time spent in quadrant P) and the mRNA levels of BDNF ($r = 0.79$, $P < 0.05$) and CREB ($r = 0.96$, $P < 0.05$; Fig. 6C and D, respectively): Animals who best recalled the platform location had a tendency to have the highest hippocampal BDNF and CREB mRNA levels.

Discussion

This study provides novel direct evidence that the cognitive enhancement seen with exercise is in fact mediated by and dependent upon the action of BDNF. Blocking hippocampal BDNF action during the exercise period was sufficient to prevent the exercise-induced enhancement in learning and memory and abrogate the exercise-induced increase in the mRNA levels of BDNF, its cognate TrkB receptor, and consummate end-products of BDNF action, i.e. synapsin I and CREB.

We found that exercise training was able to enhance learning acquisition; exercised rats receiving control injections had shorter escape latencies to find the platform than their sedentary counterparts (Fig. 2). Blocking BDNF action during exercise abolished this enhanced rate at which exercised rats acquired the MWM task; exercised animals receiving the BDNF blocker took much longer to find the platform (longer escape latencies) than exercised control animals (exc/TrkB-IgG vs. exc/cytC; Fig. 2) and their performance was not significantly different from sedentary controls (exc/TrkB-IgG vs. sed/cytC; Fig. 2). We found that exercise enhanced the recall ability of animals above sedentary control levels; exercised animals spent significantly more time in quadrant P than their sedentary counterparts (exc/cytC vs. sed/cytC; Fig. 3A–B). Blocking BDNF action during the exercise period effectively abrogated this exercise-enhanced preference for quadrant P (exc/TrkB-IgG vs. exc/cytC; Fig. 3A and F). The effect of blocking BDNF action was selective for exercise, as it did not noticeably affect the acquisition or retention abilities of sedentary animals (sed/TrkB-IgG vs. sed/cytC; Figs 2 and 3A). Inhibiting BDNF action also blocked the ability of exercise to increase downstream systems under the control of BDNF, i.e. CREB and synapsin I. In particular we found a relationship between BDNF and CREB mRNA expression and learning and memory. The results of this study indicate that exercise might utilize these BDNF-mediated systems to enhance learning acquisition and memory retention.

Exercise induces BDNF to support learning and memory

Results from BDNF levels in animals undergoing MWM training and probe trials provided the first indication that BDNF action underlies

the exercise-induced enhancement in learning acquisition and retention. Moreover, blocking BDNF action, during the exercise period prior to the MWM task, abrogated the exercise-induced increase in BDNF and TrkB mRNA levels, thereby providing direct evidence for the involvement of BDNF in learning and memory. As BDNF regulates the level of its own mRNA and that of its cognate TrkB receptor during exercise (Vaynman *et al*., 2003), blocking BDNF action during exercise might be highly successful at preventing the exercise-induced increase in BDNF and its subsequent signal transduction. This implies that the additional BDNF provided by exercise (Fig. 4A) may not be available to promote synaptic plasticity changes that underlie exercise-induced cognitive enhancement. The primary action of the BDNF blocker during the exercise period is supported by the finding that sedentary animals, that received the BDNF blocker, had similar acquisition latencies, preference for the platform quadrant and mRNA levels to sedentary animals that received the control injection (sed/TrKB-IgG vs. sed/cytC; Figs 2, 3A and 4A–D). If the action of BDNF was blocked during the MWM task, sedentary animals receiving the BDNF blocker would have performed below sedentary control levels, as depicted by the following finding. Quenching endogenous BDNF with function-blocking anti-BDNF antibodies during the MWM task impairs both spatial learning and memory in rats, to result in longer escape latencies during the training phase and poorer task completion during the probe trial (Mu *et al*., 1999; Mizuno *et al*., 2000).

Exercise affects CREB: implications for memory formation

Blocking BDNF action prevented the exercise-induced increase in CREB mRNA levels (Fig. 4D), indicating that exercise-induced BDNF levels might enhance memory consolidation on the MWM task by augmenting CREB expression. CREB functions in activity-dependent long-term neuronal plasticity and is believed to be an evolutionarily conserved molecule necessary for the formation of LTM (Dash *et al*., 1990; Bourtchouladze *et al*., 1994; Yin *et al*., 1995). For example, odour memory is impaired in Drosophila in which CREB function has been disrupted by a dominant negative CREB protein (Yin *et al*., 1995). In mice, targeted disruption of CREB isoforms results in LTM deficiency (Bourtchouladze *et al*., 1994). We found that with exercise, BDNF and CREB mRNA levels were significantly and positively associated with each other as well as with performance on the probe trial, such that animals with the highest BDNF expression also had the highest CREB expression and the best recall of the platform location (Figs 4E, and 6C and D). Blocking BDNF action during exercise training not only abrogated the exercise-induced increase in CREB mRNA (Fig. 4D) and probe trial performance (Fig. 3A), but also disrupted this BDNF/CREB association established by exercise (Fig. 4F vs. 4E). Repeated stimulation, such as would be provided by spaced training over days, leads to a sustained phosphorylation of CREB that triggers the expression of genes responsible for initiating long-term structural and functional changes at the synapse (Bailey *et al*., 1996; Dubnau & Tully, 1998; Silva *et al*., 1998). Indeed, we found that exercised animals had significantly elevated levels of the active form of CREB, i.e. p-CREB (Fig. 5). The level of CREB can act as a molecular switch to activate the transcription necessary for LTM (Yin & Tully, 1996), such that an overexpression of CREB has been shown to enhance LTM formation by reducing the usual requirements for repetition and rest during training (Yin & Tully, 1996). Similarly, exercise-induced enhancement in CREB expression, which we have also found to be significantly increased immediately after exercise (Vaynman *et al*., 2003), may enable exercised animals to recall the location of the platform with less training. Exercised animals showed a significant improvement in learning on day 3 of MWM training, as compared with days 1 and 2, whereas sedentary animals gained this improvement on day 4 of training (exc/cytC vs. sed/cytC; Fig. 2). Indeed, BDNF was both associated with CREB and learning acquisition, as the fastest learners had a tendency to have the largest increases in BDNF and associated CREB expression (Figs 4E, and 6A and B). As exercised animals given the BDNF retained the day 3 improvement in learning acquisition, possibly other molecules, such as the *N*-methyl-D-aspartate receptor, which we have previously found to interact with

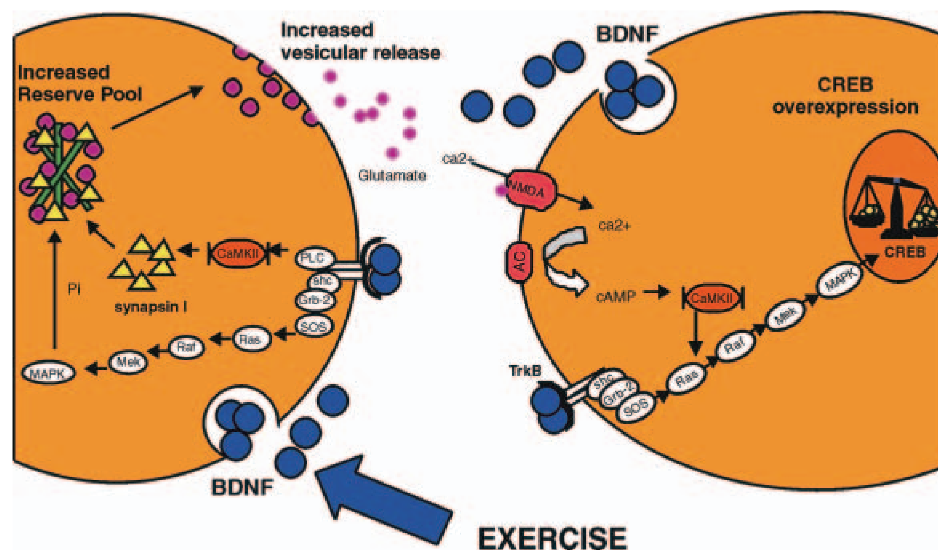

FIG. 7. Potential mechanism through which BDNF may enhance learning and memory in the hippocampus under the action of exercise. Exercise-induced BDNF levels are depicted activating signal transduction cascades that lead to synapsin I and CREB mediated plasticity. BDNF activation of its TrkB receptor may increase the synaptic reserve pool by activating a PLC-CAMK-II cascade to increase synapsin I levels. Additionally, BDNF may increase neurotransmitter release by phosphorylating synapsin I via the MAP-kinase cascade. Postsynaptically, BDNF activation of the MAP-Kinase cascade, supplemented by additionally interaction with the NMDA receptor and CAMK-II, can lead to CREB overexpression that is necessary for implementing long-term structural and functional changes supporting long-term memory.

BDNF and MAP-Kinase to regulate CREB expression (Vaynman *et al*., 2003), may participate with BDNF to mediate the effects of exercise on cognitive function (Fig. 7). It is important to remember that memory is a graded process, of which the acquisition and consolidation phases may not be separated definitively. It has been suggested that the decrease in escape latencies indicates that animals are using the recall of memories from previous experience to facilitate locating the platform (Eyre *et al*., 2003). Thus, it is possible that the facilitation of presynaptic vesicular formation and mobilization by BDNF action might translate into more long lasting changes through CREB, which is required for the stability of synaptic changes (Dash *et al*., 1990). Measuring changes in CREB expression immediately after each day of the acquisition phase of the water maze would reveal important information about this process.

We cannot completely rule out the possibility that other elements in the experimental manipulation may have contributed to the observed molecular and behavioural changes. However, in previous studies (Gomez-Pinilla *et al*., 2001) we have been able to isolate exercise as a critical factor for the increases in BDNF and CREB. When measuring these markers immediately after wheel running, there is a significant positive correlation between the amount of exercise and BDNF mRNA levels. Additionally, as in the present findings, BDNF and CREB were found to be significantly correlated with each other when measured immediate after exercise training (Gomez-Pinilla *et al*., 2001). As all animals were given a 1-week acclimation period prior to the start of the experiments, exposure to a novel environment is not likely to have contributed to the observed changes. Other factors to consider include the minimal spontaneous physical activity and deficient social environment of the sedentary control rats. These observations raise the issue as to whether the BDNF levels and associated cognitive function are 'subnormal' relative to the average sedentary human who is exposed to a relatively complex environment. In this case, one would have to consider the possibility that a rat housed in a sedentary cage is not an appropriate control condition for a variety of experiments. Accordingly, socially housed animals exposed to some moderate complexity can be a better control for future studies designed to simulate the human condition.

Exercise affects synapsin I: implications for synaptic plasticity underlying learning and memory

Blocking BDNF action prevented the exercise-induced increase in synapsin I mRNA levels, as measured after the probe trial (Fig. 4C). This result was consistent with our previous finding that synapsin I expression was dependant on BDNF action and exercise duration, when measured immediately after the exercise period (Vaynman *et al*., 2003). Given the role of synapsin I in vesicular release, these findings indicate that exercise may employ BDNF to modulate the distribution of vesicles and neurotransmitter release by regulating synapsin I. A proper distribution of vesicles and neurotransmitter release provided by functional levels of synapsin I could be important for learning, as a recent clinical study of a family of epileptics showed that a genetic mutation in the synapsin I gene is associated with learning difficulties (Garcia *et al*., 2004). Synapsin I effectively establishes a synaptic vesicle reserve pool by tethering synaptic vesicles to the actin cytoskeleton. Thereby, in the case of a synapsin I deficit, such as in SYNI mice, a marked decrease in synaptic density becomes prominent in the reserve pool (Li *et al*., 1995; Takei *et al*., 1995). Thus, one way exercise may boost learning and memory is by using a BDNF-mediated mechanism to provide a substantial reserve pool, shown to be necessary for the prevention of vesicular run-down during high frequency stimulation (Pieribone *et al*., 1995). As CAMKII has been shown to contribute to the BDNF regulation of synapsin I during exercise (Vaynman *et al*., 2003), exercise-induced BDNF levels may activate CAMKII through phospholipase C anchored to its TrkB receptor (Blanquet & Lamour, 1997) to regulate synapsin I levels (Fig. 7). With depolarization, synapsin I is phosphorylated to release synaptic vesicles from the reserve pool to the release pool (Greengard *et al*., 1993). Since inhibiting synapsin I reduces both the synaptic reserve pool and neurotransmitter release (Hilfiker *et al*., 1999), exercise may additionally boost learning and memory by using BDNF action to increase neurotransmitter release via MAP-kinase phosphorylation of synapsin I (Jovanovic *et al*., 2000; Fig. 7).

Conclusions

Our results showed that exercise enhanced spatial learning and memory on the MWM task and was associated with an increase in the mRNA levels of BDNF, its TrkB receptor, and its consummate end-products, synapsin I and CREB. Importantly, our results are novel as they provide the first direct evidence that exercise may predominately employ the action of BDNF to enhance cognitive function. Furthermore, given the extensive involvement of the BDNF system in many disorders of cognitive function, such as schizophrenia (Egan *et al*., 2003), traumatic brain injury (Horsfield *et al*., 2002), dementia (Ando *et al*., 2002), and Alzheimer's disease (Tsai *et al*., 2004), the finding that a short and moderate exercise period is sufficient to enhance learning and memory suggests that exercise is a highly accessible form of intervention that could be used in conjunction with the standard method of care.

Acknowledgements

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Abbreviations

ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; CREB, cAMP response-element-binding protein; BSA, bovine serum albumin; CytC, cytochrome C; LTM, long-term memory; LTP, long-term potentiation; MWM, Morris water maze; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction.

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