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Exercise normalizes levels of MAG and Nogo-A growth inhibitors after brain trauma

Gabriela Chytrova,[1] Zhe Ying[1] and Fernando Gomez-Pinilla[1,2]
[1]Department of Physiological Science, UCLA, 621 Charles E. Young Drive, Los Angeles, CA 90095, USA
[2]Division of Neurosurgery, UCLA Brain Injury Research Center, Los Angeles, CA 90095, USA

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Abstract

Myelin is a major obstacle for axonal growth after CNS injury, to the extent that it is crucial to develop interventions to counteract postinjury growth inhibition and foster neural repair. We have studied the effects of the fluid percussion injury (FPI) model of traumatic brain injury (TBI) on protein levels of two myelin-associated molecules, myelin-associated glycoprotein (MAG) and Nogo-A, in the adult rat. We found that FPI elevated hippocampal levels of MAG and Nogo-A. Given the beneficial effects of exercise on CNS function, we evaluated the capacity of exercise to reduce these myelin-derived proteins after FPI. One week of voluntary running wheel exercise overcame the injury-related increase in MAG and Nogo-A. The action of brain-derived neurotrophic factor (BDNF) has been associated with exercise as well as with the modulation of growth inhibition *in vitro*. We found that the selective blockade of BDNF using the immunoadhesive chimera TrkB-IgG abolished the effects of exercise on MAG and Nogo-A. FPI reduced levels of growth-associated protein 43 (GAP-43), a marker of axonal growth, and synaptophysin (SYP), an indicator of synaptic growth. Exercise counteracted the effects of FPI on GAP-43 and SYP, while BDNF blockade abolished these effects of exercise. Protein kinase A (PKA) has been related to the ability of BDNF to overcome growth inhibition. In agreement, we found that exercise increased PKA levels and this effect was prevented by BDNF blockade. These results indicate that exercise promotes a permissive cellular environment for repair after TBI, in a process in which BDNF plays a central role.

Introduction

Central nervous system (CNS) injuries promote a hostile environment for neurite outgrowth with subsequent consequences for the regenerative potential of neurons (Filbin, 2003; Spencer *et al*., 2003). Specific myelin components, such as myelin-associated glycoprotein (MAG) and Nogo-A, provide a major source of inhibition for growing neurites after CNS injury (Filbin, 2003; Lenzlinger *et al*., 2005). MAG is a member of the immunoglobulin-like family and is expressed in oligodendrocytes (Salzer *et al*., 1987; Trapp *et al*., 1989; Chaudhry & Filbin, 2007). Nogo-A belongs to the reticulon family of proteins and is expressed in both oligodendrocytes and neurons (Grandpre *et al*., 2000; Wang *et al*., 2002; Hunt *et al*., 2003). Many of the studies to date directed at understanding the mechanisms involved in injury-induced growth inhibition have been performed in the spinal cord. However, there are several recent studies that address the effects of injury on myelin growth-inhibitory molecules in animal models of traumatic brain injury (TBI; Dubreuil *et al*., 2006; Marklund *et al*., 2006; Thompson *et al*., 2006). Growth inhibitor function and expression has also been extensively studied in ischemic stroke injury models (Zhou *et al*., 2003; Irving *et al*., 2005; Erb *et al*., 2006; Eslamboli *et al*., 2006; Weinmann *et al*., 2006). In turn, TBI is an important health issue for the world population, and there is a lack of effective therapies to reduce injury-related disorders.

Correspondence: Dr Fernando Gomez-Pinilla, [1]Department of Physiological Science, as above.
E-mail: Fgomezpi@ucla.edu

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Several molecular systems that have the capacity to modulate the growth-inhibitory function of MAG and Nogo-A have been identified. In particular, brain-derived neurotrophic factor (BDNF) is gaining recognition as a main modulator of MAG and Nogo-A inhibition. For example, *in vitro* studies have shown that neurons stimulated with BDNF have an enhanced ability to counteract the effects of myelin-associated inhibitors through a mechanism involving the action of protein kinase A (PKA; Cai *et al*., 1999; Gao *et al*., 2003). The results of these studies promote the notion that elevated production of BDNF could help the CNS overcome its resistance to regeneration. However, the invasive nature of the current methodologies for delivering BDNF into the CNS precludes profiting from the benevolent action of BDNF (Hicks *et al*., 1999; Blaha *et al*., 2000; Griesbach *et al*., 2007). Exercise has emerged as a physiological noninvasive strategy for elevating BDNF production in select regions of the brain and spinal cord (Gomez-Pinilla *et al*., 2002; Vaynman *et al*., 2003). It is known that physical activity increases axonal growth after nerve injury and induces the expression of important indicators of axonal and synaptic growth, growth-associated protein-43 (GAP-43) and synaptophysin (SYP; Gomez-Pinilla *et al*., 2002; Molteni *et al*., 2004; Vaynman *et al*., 2006), but it remains to be determined whether exercise can help the regenerative events by modulating the action of axonal growth inhibitors.

The present study was designed to investigate the effects of TBI on growth-inhibitory systems such as MAG and Nogo-A, and the capacity of voluntary exercise to modulate the effects of the injury. We have used lateral fluid percussion injury (FPI) as an animal model of human concussive injury (McIntosh *et al*., 1989; Morales *et al*., 2005; Thompson *et al*., 2005), allowing an in-depth analysis of the

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molecular changes occurring in the injured brain. These studies have been focused on the potential of BDNF to mediate the effects of exercise on growth inhibition. Overall, our findings support the notion that voluntary exercise positively influences the regenerative ability of the injured CNS by reducing growth-inhibitory molecules.

Materials and methods

The experiments were performed in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animals were continually monitored and all procedures were approved by the UCLA Chancellor's Animal Research Committee.

Fluid percussion injury

Adult male Sprague–Dawley rats ($n = 48$; Charles River Laboratories, Inc., Wilmington, MA, USA), $\sim$2 months of age, were acclimatized in the receiving facility prior to the beginning of the study. All rats were housed in standard polyethylene cages in an environmentally controlled room (22–24 °C) with a 12-h light–dark cycle. Rats (300–350 g) were exposed to mild FPI or sham injury. FPI was performed as previously described (Wu *et al*., 2004). In brief, rats were anaesthetized with isoflurane and secured in a stereotaxic apparatus. With the aid of a microscope (Wild, Heerburg, Switzerland), a 3.0-mm-diameter craniotomy was made with a high-speed drill (Dremel, Racine, WI, USA), 3.0 mm posterior to the bregma and 6.0 mm lateral to the midline. A plastic injury cap was attached to the skull over the craniotomy with silicone adhesive and dental cement. After the dental cement had completely hardened, the cap was filled with 0.9% saline solution. To ensure that all animals were in an identical plane of anaesthesia at the time of injury, the injury cap of each anaesthesized rat was attached to the fluid percussion device and a paw pinch was performed once every 15 s until a hind-limb withdrawal was observed. To apply mild FPI, a standard fluid percussion pulse of 1.5 atm was administered immediately after the first withdrawal to a paw pinch. The fluid percussion pulse was applied on the left side to be consistent with our previous TBI experiments (Wu *et al*., 2003, 2004). TBI lesion after FPI has been described in our previous publication (Wu *et al*., 2003). We chose FPI of mild severity because it produces dysfunction in molecular systems without having any effect on neuronal death (Lyeth *et al*., 1990; Wu *et al*., 2003). No Fluoro-Jade B-stained cells (an indicator of neurodegeneration) were found in the hippocampus of any FPI-treated rats (Wu *et al*., 2003).

The duration of apnea and unconsciousness were recorded as the elapsed time from the injury to resumed spontaneous breathing and a paw pinch response, respectively. There was no mortality due to FPI, and apnea ($\sim$ 10 s) and paw pinch (90–115 s) response times were similar among the animals. Immediately after the delivery of the fluid percussion pulse, the injury cap was removed under anaesthesia and the incision was closed with sutures and covered with neomycin antibiotic cream. The rats were placed in a heated recovery chamber for $\sim$ 1 h before being returned to their cages. A sham-injury group of rats received the same treatment except for the final FPI. We have performed pilot studies to determine the timing of the response for the effects of TBI on MAG and Nogo-A. Our previous findings also showed that 1 week of voluntary exercise significantly increased BDNF in the hippocampus (Vaynman *et al*., 2003). MAG and Nogo-A were thus assessed 10 days post-TBI to determine whether 1 week of exercise can modulate postinjury levels of MAG and Nogo-A.

Drugs and preparation of microbeads

Recombinant human TrkB-IgG chimera (R & D System, Minneapolis, MN, USA), comprising the extracellular BDNF binding domain of human TrkB and the Fc domain of IgG, was used as a highly potent and specific antagonist of BDNF (Shelton *et al*., 1995; Vaynman *et al*., 2006). Although the primary target of the TrkB-IgG chimera is to block BDNF function, we do not exclude the possibility that other molecules may be affected by this treatment. Fluorescent latex microbeads (LumaFluor, Naples, FL, USA) were used as the vehicle for drug insertion into the hippocampus. TrkB-IgG in powder was dissolved in sterile phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) to prepare a stock solution (100 μg/mL). Microbeads were prepared by the previously described methods (Riddle *et al*., 1997; Lom & Cohen-Cory, 1999). This consisted of coating the microbeads with TrkB-IgG 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). 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

Rats were randomly divided into FPI, sham, exercise and sedentary groups, treated with TrkB-IgG or saline, to generate the following groups ($n = 6$ rats per group): FPI/saline/sedentary, FPI/saline/exercise, FPI/TrkB-IgG/sedentary, FPI/TrkB-IgG/exercise, sham/saline/sedentary, sham/saline/exercise, sham/TrkB-IgG/sedentary, and sham/TrkB-IgG/exercise. On day 3 after FPI or sham surgery, the animals received an injection of TrkB-IgG embedded in microbeads ipsilateral to the lesion. We have previously used the microbead injection method to successfully block the action of BDNF with TrkB-IgG in the hippocampus (Vaynman *et al*., 2006). Microbeads embedded in saline were used as a control. We found no effect of microbeads alone on any of the molecules that were analysed (Vaynman *et al*., 2003; Vaynman *et al*., 2006). Other studies have also reported successful delivery of bioactive agents using microbeads without any side-effects when using microbeads alone (Riddle *et al*., 1997; Lom & Cohen-Cory, 1999). The microbead injection was administered early in the morning to provide an ample recovery time for all animals to begin running that same evening. The rats were anaesthetized with isoflurane and secured in a stereotaxic apparatus to measure the location for the injection. TrkB-IgG (5 μg/μL) or 0.9% saline was injected into the left hippocampus (3.8 mm posterior to the bregma, 2.6 mm lateral to the midline, and 3.7 mm vertically) using a Hamilton syringe in a volume of 2.0 μL over 15 min. The location of the microbead injection was verified by fluorescence microscopy (Fig. 1E), using an Olympus BX51 microscope.

Exercise paradigm

All rats were housed in standard polyethylene cages in an environmentally controlled room (22–24 °C) with a 12 h light–dark cycle. The animals in the exercise groups were transferred into individual cages where they had a consistent access to the running wheel (diameter 31.8 cm, width 10.0 cm) 3 days after the sham surgery or FPI. The running wheel rotated freely against a resistance of 100 g attached to a receiver that monitored revolutions every hour (VitalViewer Data Acquisition System software, Mini Mitter, Sunriver, OR, USA). The rats were allowed to exercise *ad libitum* for seven consecutive days. According to our previous observations, the animals

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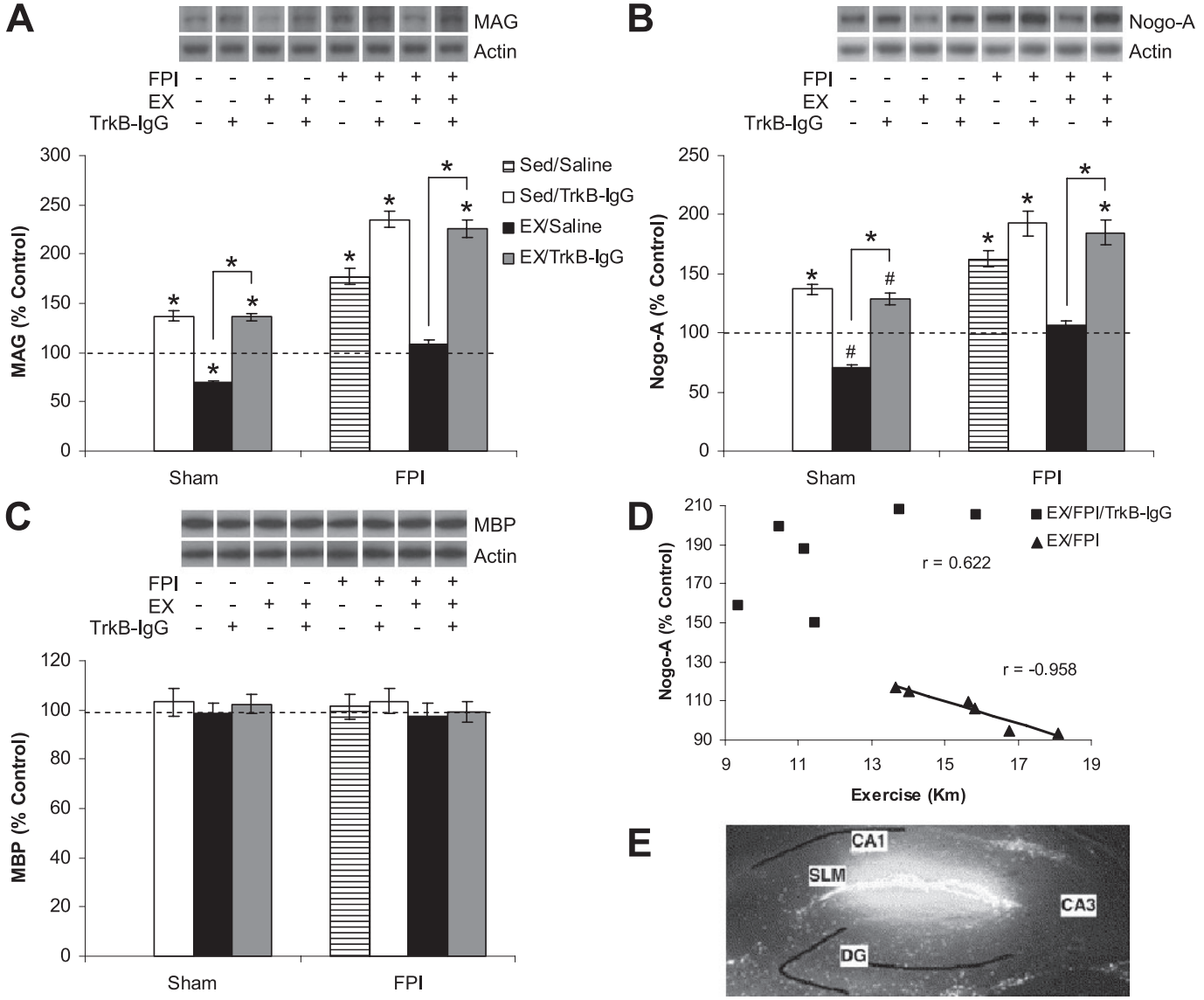

FIG. 1. Effects of blocking the action of BDNF on MAG, Nogo-A and MBP levels in the hippocampus. Exercise decreased levels of (A) MAG and (B) Nogo-A in sham (Sham/EX/Saline) and FPI (FPI/EX/Saline) animals. Sequestering BDNF in the hippocampus with TrkB-IgG prevented the effects of exercise on MAG and Nogo-A levels in both sham (Sham/EX/TrkB-IgG) and FPI (FPI/EX/TrkB-IgG) animals. (C) MBP levels were not affected following TrkB-IgG treatment in any of the experimental groups. The values were converted to percentages of Sham/Sed/Saline controls represented by the horizontal dashed line (mean ± SEM; $^{\#}P < 0.01$, $^{*}P < 0.001$). FPI, fluid percussion injury; EX, exercise. (D) TrkB-IgG treatment abolished a negative correlation between Nogo-A (FPI/EX/TrkB-IgG) and exercise distance. (E) Tissue section in the coronal plane of the hippocampus showing the microbead injection site in the stratum lacunosum moleculare (SLM) using fluorescence microscopy. Highlighted areas represent high fluorescence activity associated with the microbead injection of TrkB-IgG. For reference, hippocampal areas cornus amonus 1 (CA1), CA3 and dentate gyrus (DG) have been labelled.

generally run the most at night. Therefore, TrkB-IgG treatment was performed early in the morning to provide sufficient recovery time from anaesthesia for the rats to be alert by the evening. The animals recovered from isoflurane anaesthesia within 1 h after the surgery and started running at night. Animals were killed the morning after the last running period.

Tissue preparation and protein determination

The entire hippocampus on the FPI-treated side of the brain was collected upon decapitation for protein determination. Tissue was collected into 1.5-mL Eppendorf tubes, immediately frozen on dry ice and stored at −70 °C. Hippocampi ipsilateral to the craniotomy were homogenized in a freshly prepared lysis buffer (NaCl, 137 mM; Tris-HCl pH 8.0, 20 mM; NP-40, 1%; glycerol, 10%; phenylmethylsulphonyl fluoride, 1 mM; aprotinin, 10 μg/mL; leupeptin, 1 μg/mL; and sodium vanadate, 0.5 mM). Homogenates were centrifuged at 12 000 ***g*** for 20 min to remove insoluble material. The supernatants were collected into clean 1.5-mL tubes, frozen on dry ice and stored at −70 °C. The total protein concentration of hippocampal homogenates was determined with a MicroBCA kit (Pierce, Rockford, IL, USA), using BSA as a standard. BDNF protein was quantified using an enzyme-linked immunosorbent assay (BDNF Emax ImmunoAssay System Kit, Promega, Madison, WI, USA). Unknown BDNF concentrations were compared with known BDNF concentrations using a calibration curve.

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Western blot

Relative levels of MAG (100 kDa), Nogo-A (220 kDa), myelin basic protein (MBP; 18 kDa), PKA (41 kDa), GAP-43 (43 kDa) and SYP (38 kDa) were analysed by Western blot. Equal amounts (25 μg) of protein samples were separated by electrophoresis on a 10% polyacrylamide gel and electrotransferred to an Immobilon-P transfer membrane (Millipore, Bedford, MA, USA). Nonspecific binding sites were blocked with 5% nonfat milk in TBS buffer with 0.1% Tween-20 (pH 7.6). Membranes were incubated with the following primary antibodies: anti-MAG (1 : 4000; 100 kDa; Chemicon International), anti-Nogo-A (1 : 5000; 220 kDa; Santa Cruz Biotechnology; SC-11032), anti-MBP (1 : 1000; 18 kDa; Chemicon International), anti-PKA (1 : 6000; 41 kDa; Upstate), anti-GAP-43 (1 : 2000; 43 kDa; Santa Cruz Biotechnology), anti-SYP (1 : 50 000; 38 kDa; Santa Cruz Biotechnology), and antiactin (1 : 2000; 43 kDa; Santa Cruz Biotechnology) followed by antimouse IgG horseradish peroxidase conjugate for MAG and MBP, antigoat IgG horseradish peroxidase conjugate for Nogo-A, GAP-43 and actin or antirabbit IgG horseradish peroxidase conjugate for PKA and SYP (Santa Cruz Biotechnology). After rinsing with buffer (0.1% Tween-20 in TBS), the immunocomplexes were visualized by chemiluminescence using the Amersham ECL Plus Western Blotting Detection kit (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) according to the manufacturer's instructions. The film signals were digitally scanned using an HP Scanner (HP Scanjet 3970) and quantified with NIH Image software, normalized for actin levels.

Immunohistochemistry

Additional rats ($n = 16$) from sham/sedentary, sham/exercise, FPI/sedentary and FPI/exercise groups (with and without TrkB-IgG treatment) were injected with a lethal dose of Nembutal (75 mg/kg i.p.), then intracardially perfused with PBS (pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and 20% sucrose in 4% paraformaldehyde. Serial coronal brain sections (25 μm) were cut on a cryostat, collected free-floating in PBS and processed for immunohistochemistry, as previously described (Gomez-Pinilla *et al.*, 1992; Vaynman *et al.*, 2004). Tissue sections were incubated overnight at room temperature in a solution containing the mouse anti-MAG monoclonal primary antibody (1 : 200; Chemicon International) diluted in PBS, 2% BSA and 0.1% Triton X-100. Immunohistochemistry controls were performed by omission of the primary antibody. The results of immunohistochemistry controls were negative as no staining was observed in cell structures. Analysis was restricted to the hippocampus based on our previous studies showing significant effects of TBI and exercise on hippocampal plasticity by regulating BDNF (Wu *et al.*, 2003; Vaynman *et al.*, 2004). In our previous report we found no hippocampal damage due to cell death after mild TBI (Wu *et al.*, 2003). Fluoro-Jade B staining that selectively labels degenerating neurons (Schmued & Hopkins, 2000; Kubova *et al.*, 2001) revealed no Fluoro-Jade B-labelled cells in the hippocampus of injured animals (Wu *et al.*, 2003).

Statistical analyses

The mean protein levels were calculated for each group ($n = 6$ rats per group). ANOVA and Fisher tests (Statview software, Abacus Concepts, CA, USA) were used to determine any significant differences among the groups at $P < 0.05$. The results were expressed as mean percentages of control values and are presented as the mean ± SEM. A linear regression analysis was performed on individual samples to evaluate association between variables (running distance with protein levels, GAP-43 with MAG or Nogo-A, and SYP with MAG or Nogo-A).

Results

Exercise normalized levels of MAG and Nogo-A increased after injury

We measured levels of MAG and Nogo-A in the hippocampus ipsilateral to the injury 10 days following lateral FPI to determine whether brain trauma alters expression of myelin growth-inhibitory molecules. FPI increased MAG and Nogo-A levels to 177.2% (FPI/Sed/Saline; $P < 0.001$; Fig. 1A) and 162.4% (FPI/Sed/Saline; $P < 0.001$; Fig. 1B), respectively, compared to sham-operated controls ($n = 6$ per group). We then evaluated the capacity of 1 week of voluntary exercise to counteract the effects of FPI on the hippocampal levels of MAG and Nogo-A. Results showed that voluntary exercise abolished the injury-induced up-regulation of MAG (from 177.2 to 108.3%; $P < 0.001$; Fig. 1A) and Nogo-A (from 162.4 to 106.0%; $P < 0.001$; Fig. 1B) as levels of these factors returned to control values.

Exercise also had significant effects on MAG and Nogo-A levels in sham animals. MAG was reduced by 29.8% (Sham/EX/Saline; $P < 0.001$; Fig. 1A) and Nogo-A was decreased by 29.1% (Sham/EX/Saline; $P < 0.001$; Fig. 1B) after exercise. On the other hand, no changes in the levels of MBP, a major component of myelin, were found in any experimental group (Fig. 1C), suggesting that the effects of injury and exercise described above were specific for MAG and Nogo-A. Additionally, the normal variability in the total running distance among the animals showed that levels of the myelin inhibitors for individual animals were negatively correlated with the running distance. In both FPI-treated and sham-operated groups, the animals that ran the most had the lowest MAG (FPI: $r = -0.959$, $P < 0.001$; Sham: $r = -0.933$, $P < 0.001$; data not shown) and Nogo-A (FPI: $r = -0.958$, $P < 0.001$; Fig. 1D; Sham: $r = -0.960$, $P < 0.001$; data not shown) levels. Furthermore, in separate groups of animals, immunohistochemical staining for MAG showed a qualitative increase in immunoreactivity in oligodendrocyte-like cells in stratum lucidum and stratum pyramidale of cornus amonus 3 (CA3) and stratum lacunosum moleculare of CA1 regions of the hippocampus after FPI, which was normalized after exercise (Fig. 2A–D). Expression of MAG and Nogo-A in sham animals may reflect their normal function in the maintenance of myelin, and Nogo-A expression may also be important for regulation of neuronal function.

BDNF blockade counteracted the effect of exercise on MAG and Nogo-A

Based on our previous reports that voluntary exercise increases BDNF expression and the present findings that exercise reduces myelin growth-inhibitory molecules in the hippocampus, we tested the role of BDNF in mediating the effects of exercise on MAG and Nogo-A. Animals received TrkB-IgG injection in the lesion-side hippocampus 3 days after FPI or sham surgery, and were exposed to exercise for 1 week after the injection. Sham-operated sedentary animals ($n = 6$) received a saline injection and served as controls. MAG and Nogo-A levels were measured in the hippocampus 1 week after TrkB-IgG or saline injection. BDNF blockade abolished the exercise-induced down-regulation of MAG and Nogo-A (EX/Saline vs. EX/TrkB-IgG; Fig. 1A and B). Blocking BDNF action in FPI animals (FPI/EX/TrkB-IgG) effectively prevented the reduction of MAG

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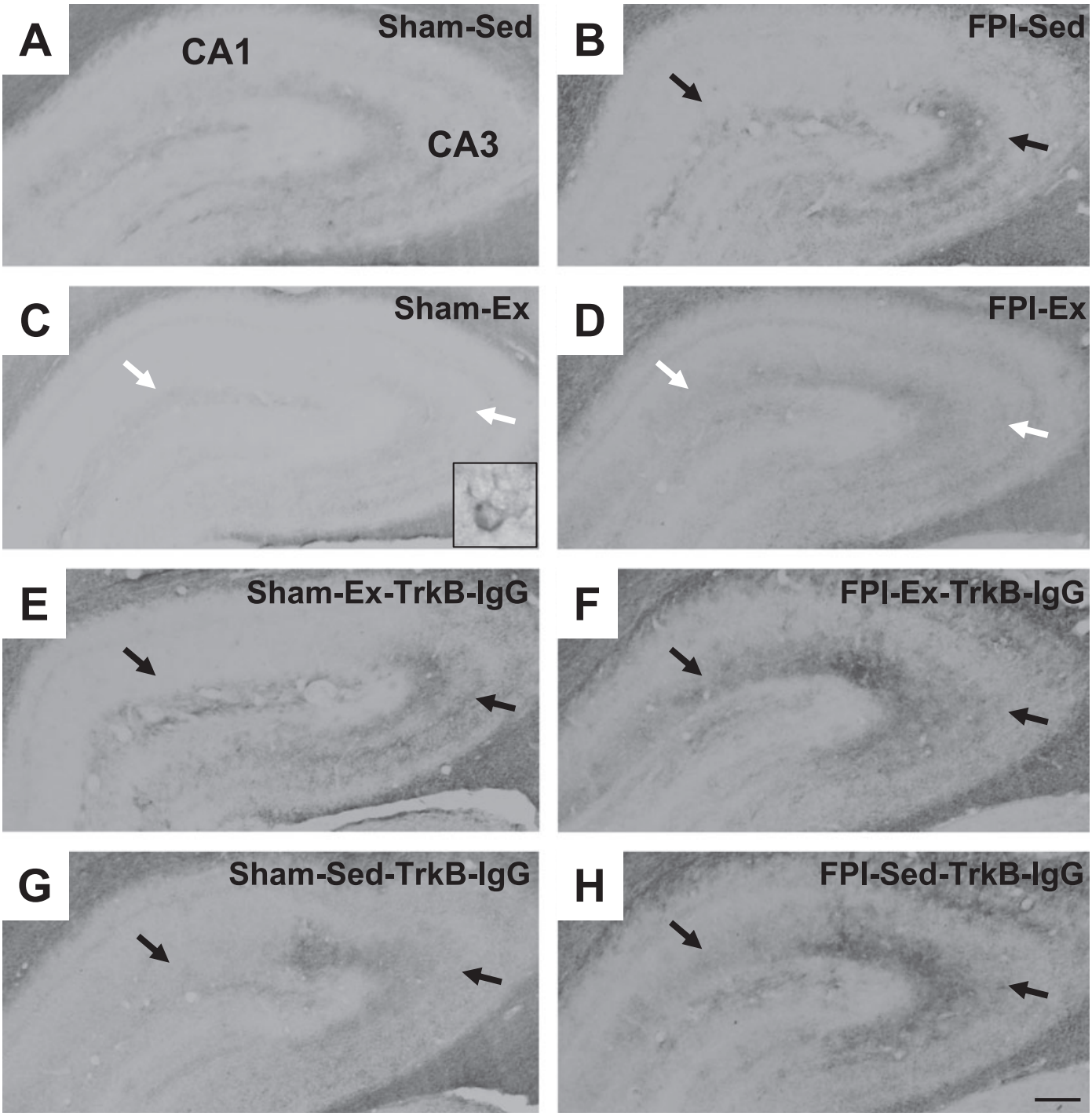

FIG. 2. Immunohistochemistry for MAG in coronal sections of the hippocampus after FPI and/or exercise. (A–D) Representative sections show MAG immunoreactivity in (A) sedentary, (B) injury, (C) exercise and (D) injury/exercise rats. Insert in C shows a high magnification of a MAG-positive cell from the CA3 region of an exercise rat. Black arrows denote the marked increase in MAG immunoreactivity in CA3 and CA1 regions in (B) a sedentary injured rat, while white arrows show the reducing effect of exercise on MAG immunoreactivity in (C) a sham-injured rat and (D) a brain-injured rat. (E–H) Effects of BDNF blockade on immunoreactivity for MAG of sham-injured or brain-injured rats. Blocking BDNF function with TrkB-IgG eliminated the reducing effect of exercise on MAG immunoreactivity in (E) sham and (F) FPI rats and increased MAG immunolabel in (G) sham and (H) FPI sedentary rats. FPI, fluid percussion injury; Sed, sedentary; Ex, exercise. Scale bar, 250 μm.

and Nogo-A induced by exercise, by increasing MAG levels to 225.3% (Fig. 1A; $P < 0.001$) and Nogo-A levels to 184.9% (Fig. 1B; $P < 0.001$) of the control group. BDNF blockade in sham animals (Sham/EX/TrkB-IgG) also inhibited the effects of exercise by increasing MAG levels to 136.2% (Fig. 1A; $P < 0.001$) and Nogo-A levels to 128.4% (Fig. 1B; $P < 0.01$) of the control group. Similarly, TrkB-IgG treatment blocked the effect of exercise on reducing MAG immunoreactivity in both FPI and sham animals (Fig. 2A–H). Additionally, BDNF blockade eliminated the negative correlation between levels of MAG (FPI: $r = 0.726$, $P > 0.05$; Sham: $r = 0.646$,

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$P > 0.05$; data not shown) and Nogo-A (FPI: $r = 0.622$, $P > 0.05$; Fig. 1D; Sham: $r = 0.662$, $P > 0.05$; data not shown) and the running distance.

In sedentary animals, TrkB-IgG treatment after FPI increased MAG and Nogo-A levels compared to the saline-treated controls after FPI from 177.2 to 235.2% (FPI/Sed/Saline vs. FPI/Sed/TrkB-IgG; Fig. 1A) and 162.4 to 192.7% (FPI/Sed/Saline vs. FPI/Sed/TrkB-IgG; Fig. 1B), respectively. This increase in myelin growth inhibitors may reflect the involvement of BDNF in regulating their postinjury levels as BDNF blockade with TrkB-IgG increased MAG and Nogo-A even more compared to a saline-treated group after FPI. In sham animals (Sham/Sed/TrkB-IgG), TrkB-IgG treatment alone significantly increased MAG and Nogo-A to 137.3% (Fig. 1A; $P < 0.001$) and 136.8% (Fig. 1B; $P < 0.001$), respectively, compared to the control group (Sham/Sed/Saline). Importantly, blocking BDNF action did not affect the levels of the myelin component, MBP, in any of the experimental groups (Fig. 1C), suggesting specificity for BDNF-mediated effects of exercise on MAG and Nogo-A.

Exercise acted on BDNF to counteract an injury-related reduction in proteins important for growth and synaptic plasticity

Given the demonstrated involvement of BDNF and PKA on overcoming the inhibitory effects of myelin, we assessed the levels of BDNF and PKA in our paradigm. Voluntary exercise increased BDNF and PKA levels in sham animals by 47.6% (Fig. 3A; $P < 0.001$) and 25.8% (Fig. 3B; $P < 0.001$), respectively, compared to sedentary controls. On the other hand, FPI reduced BDNF and PKA levels by 31.2% (Fig. 3A; $P < 0.001$) and 31.9% (Fig. 3B; $P < 0.001$), respectively, compared to sham controls. Importantly, exercise abolished the effects of FPI on the reductions in BDNF (from 68.8 to

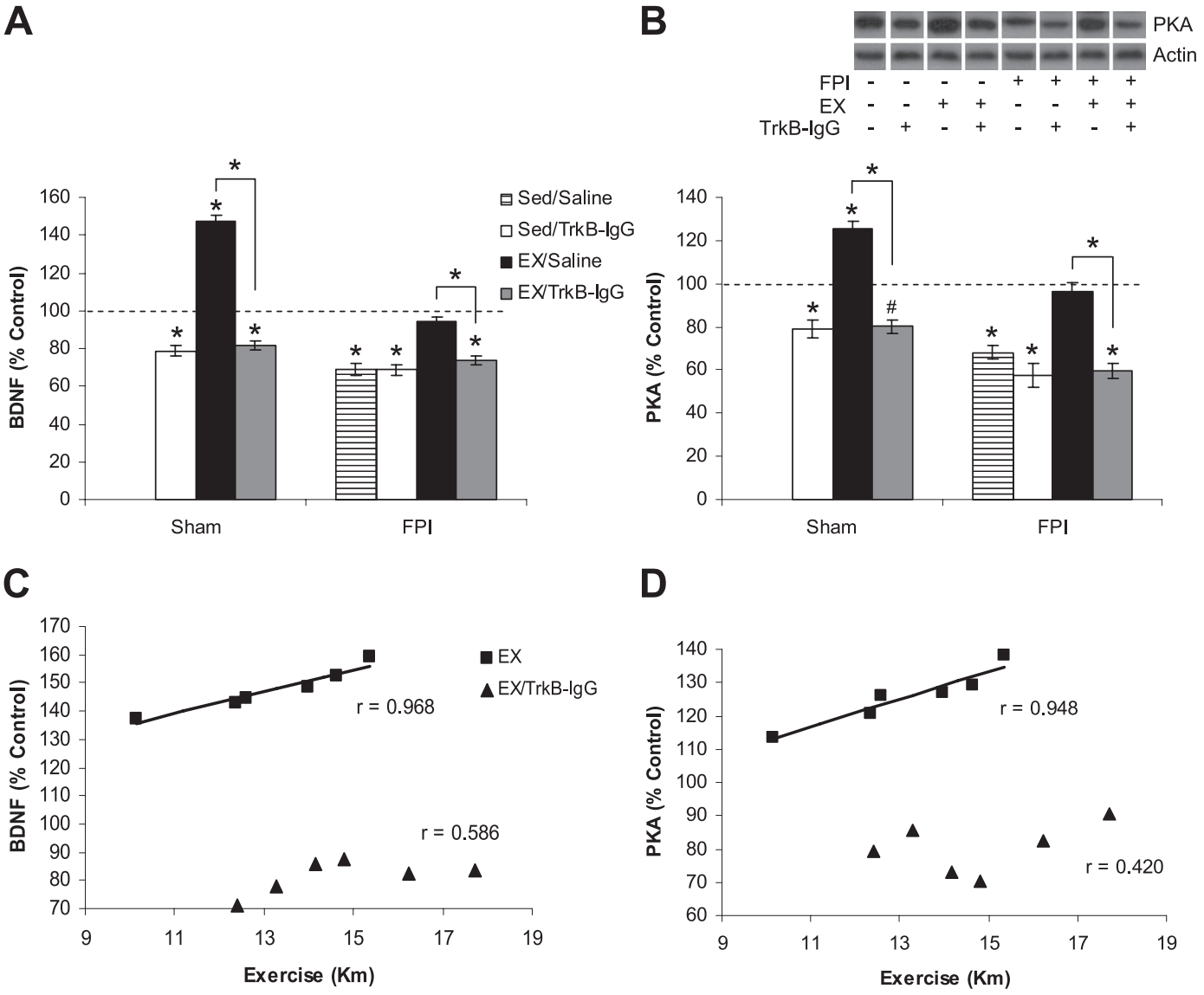

FIG. 3. Effects of blocking the action of BDNF on BDNF and PKA levels in the hippocampus. Exercise increased levels of (A) BDNF and (B) PKA in sham (Sham/EX/Saline) and FPI (FPI/EX/Saline) animals. Hippocampal TrkB-IgG application prevented the effects of exercise on (A) BDNF and (B) PKA levels in both sham (Sham/EX/TrkB-IgG) and FPI (FPI/EX/TrkB-IgG) animals. The values were converted to percentages of Sham/Sed/Saline controls represented by the horizontal dashed line (mean ± SEM; $^{\#}P < 0.01$, $^{*}P < 0.001$). TrkB-IgG treatment (EX/TrkB-IgG) in sham animals abolished a positive correlation between (C) BDNF or (D) PKA and exercise distance (EX). FPI, fluid percussion injury; EX, exercise.

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94.2%; Fig. 3A) and PKA (from 68.1 to 96.2%; Fig. 3B). Additionally, levels of BDNF (FPI: $r = 0.978$, $P < 0.001$; data not shown; Sham: $r = 0.968$, $P < 0.001$; Fig. 3C) and PKA (FPI: $r = 0.926$, $P < 0.001$; data not shown; Sham: $r = 0.948$, $P < 0.001$; Fig. 3D) increased in proportion to the amount of exercise in both FPI-treated and sham groups.

Blockade of the action of BDNF with TrkB-IgG abrogated the exercise-induced up-regulation of BDNF and PKA (EX/Saline vs. EX/TrkB-IgG; Fig. 3A and B). BDNF blockade in FPI animals (FPI/EX/TrkB-IgG) prevented the increase in BDNF and PKA induced by exercise, by reducing their levels to 73.6% (Fig. 3A; $P < 0.001$) and 59.4% (Fig. 3B; $P < 0.001$), respectively, compared to the control group. BDNF blockade in sham animals (Sham/EX/TrkB-IgG) also eliminated the effects of exercise by decreasing BDNF and PKA levels to 81.3% (Fig. 3A; $P < 0.001$) and 80.3% (Fig. 3B; $P < 0.01$), respectively, compared to the control group.

In sedentary animals, TrkB-IgG treatment decreased BDNF levels to 68.7% in FPI-treated group (FPI/Sed/TrkB-IgG) and to 78.7% in sham-operated group (Sham/Sed/TrkB-IgG) compared to the control group (Fig. 3A; $P < 0.001$). PKA levels also reduced to 57.5% in the FPI group (FPI/Sed/TrkB-IgG) and to 78.9% in the sham group (Sham/Sed/TrkB-IgG) compared to the control group (Fig. 3B; $P < 0.001$). Furthermore, TrkB-IgG treatment abolished the positive correlation between levels of BDNF (FPI: $r = 0.728$, $P > 0.05$; data not shown; Sham: $r = 0.586$, $P > 0.05$; Fig. 3C) and PKA (FPI: $r = -0.458$, $P > 0.05$; data not shown; Sham: $r = 0.420$, $P > 0.05$; Fig. 3D) and the amount of exercise in both FPI and sham groups.

BDNF blockade abolished the exercise-induced changes in levels of growth-promoting and synaptic proteins

To determine whether exercise can elevate the capacity of the injured brain for axonal growth and synaptic plasticity, we measured levels of the axonal growth marker GAP-43 and the synaptic marker synaptophysin (SYP). Exercise elevated levels of GAP-43 and SYP in sham animals by 46.7% (Fig. 4A; $P < 0.001$) and 64.8% (Fig. 4B;$P < 0.001$), respectively, compared to sedentary controls.

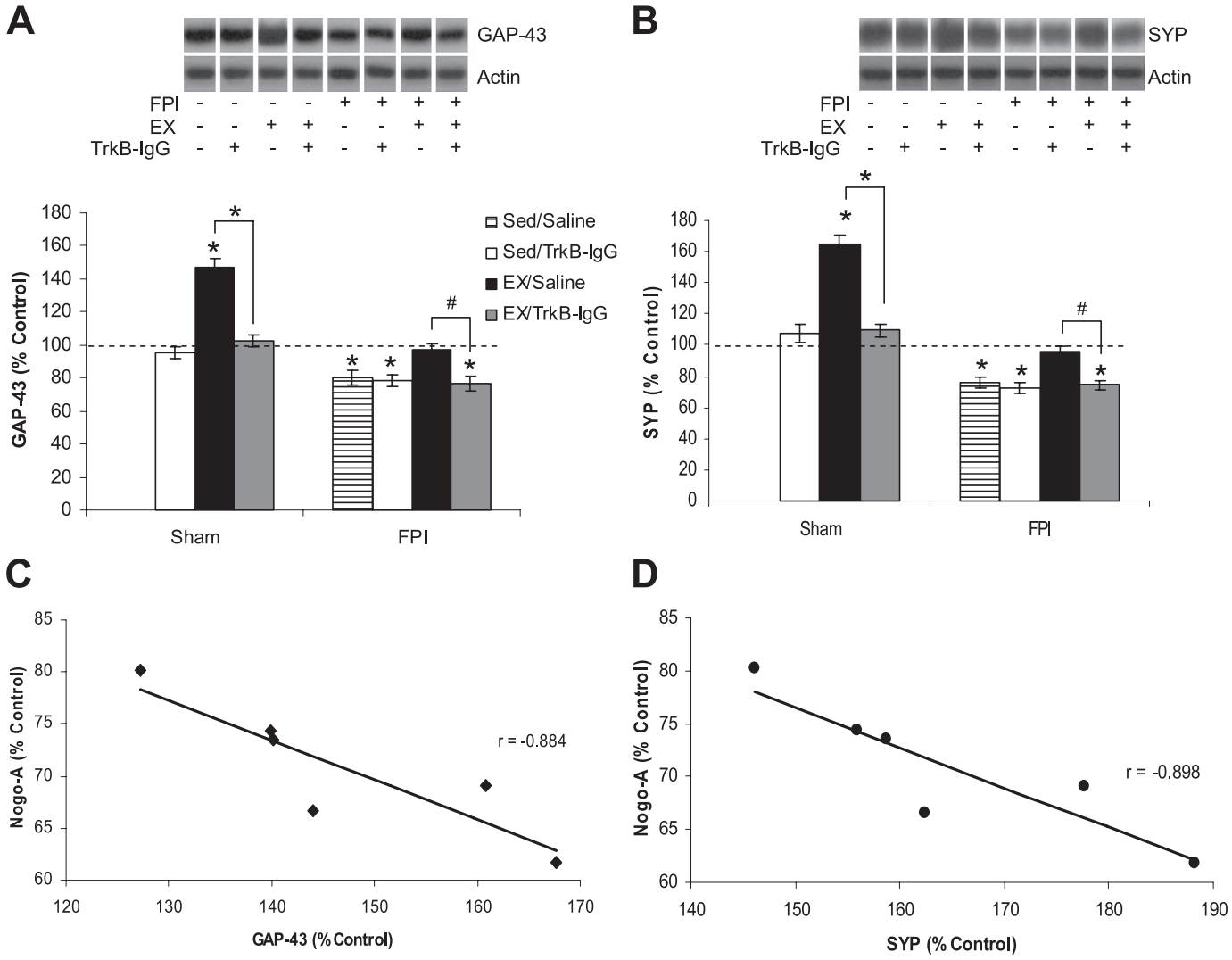

FIG. 4. Effects of blocking the action of BDNF on GAP-43 and SYP levels in the hippocampus. Exercise increased the levels of (A) GAP-43 and (B) SYP in sham (Sham/EX/Saline) and FPI (FPI/EX/Saline) animals. Blocking the action of BDNF prevented the effects of exercise on (A) GAP-43 and (B) SYP levels in both sham (Sham/EX/TrkB-IgG) and FPI (FPI/EX/TrkB-IgG) animals. The values were converted to percentages of Sham/Sed/Saline controls represented by the horizontal dashed line (mean ± SEM; $^{\#}P < 0.01$, $^{*}P < 0.001$). FPI, fluid percussion injury; EX, exercise. (C) GAP-43 and (D) SYP levels increased after exercise were negatively correlated with Nogo-A levels in sham animals.

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In contrast, FPI decreased hippocampal levels of GAP-43 and SYP by 19.7% (Fig. 4A; $P < 0.001$) and 24.2% (Fig. 4B; $P < 0.001$), respectively, compared to sham controls. Voluntary exercise abolished the effects of FPI on the reduction of GAP-43 (from 80.3 to 96.8%; Fig. 4A) and SYP (from 75.8 to 96.2%; Fig. 4B). Additionally, GAP-43 level increases after exercise were correlated with decreasing levels of MAG (FPI: $r = -0.957$, $P < 0.001$; Sham: $r = -0.813$, $P < 0.02$; data not shown) and Nogo-A (FPI: $r = -0.965$, $P < 0.001$; data not shown; Sham: $r = -0.884$, $P < 0.01$; Fig. 4C) in both FPI-treated and sham-operated animals. Similarly, exercise-enhanced SYP levels were negatively correlated with levels of MAG (FPI: $r = -0.980$, $P < 0.001$; Sham: $r = -0.792$, $P < 0.05$; data not shown) and Nogo-A (FPI: $r = -0.977$, $P < 0.001$; data not shown; Sham: $r = -0.898$, $P < 0.01$; Fig. 4D).

The exercise-induced up-regulation of GAP-43 and SYP described above was abolished after TrkB-IgG treatment in both FPI and sham animals (EX/Saline vs. EX/TrkB-IgG; Fig. 4A and B). BDNF blockade prevented the effects of exercise in injured animals (FPI/EX/TrkB-IgG) by decreasing GAP-43 and SYP levels to 76.8% (Fig. 4A; $P < 0.001$) and 74.2% (Fig. 4B; $P < 0.001$), respectively, compared to the control group. Blocking BDNF action in injured animals during the sedentary condition (FPI/Sed/TrkB-IgG) also significantly reduced GAP-43 and SYP levels to 78.4% (Fig. 4A; $P < 0.001$) and 72.4% (Fig. 4B; $P < 0.001$), respectively, compared to the control group. These results suggest that the effects of exercise on GAP-43 and SYP after brain trauma may be mediated by BDNF.

Discussion

The adult CNS has a very limited capacity for regeneration and this is largely attributable to the presence of cellular substrates unsuitable for growth. Both *in vitro* and *in vivo* evidence supports the ability of MAG and Nogo-A to inhibit neurite outgrowth (Spencer *et al*., 2003; Schwab, 2004; Buchli & Schwab, 2005; Filbin, 2006). Here we show the potential of voluntary exercise to counteract the effects of TBI on these growth inhibitors in the adult hippocampus. The application of voluntary exercise shortly after TBI normalized levels of MAG and Nogo-A as well as those of the axonal growth and synaptic markers GAP-43 and SYP. These effects of exercise were abolished by the application of a BDNF function inhibitor, TrkB-IgG, possibly in a PKA-dependent mechanism. The present results portray BDNF as an important player in the effects of exercise on reducing growth-inhibitory molecules and increasing growth-promoting factors. The overall findings emphasize the potential therapeutic action of exercise to elevate the capacity of the injured brain for plasticity and repair.

Brain trauma increased the growth-inhibitory substrate of the brain

Enhanced inhibition of regeneration after brain trauma may be associated with injury-induced up-regulation of growth-inhibitory molecules. Our results show that levels of MAG and Nogo-A were increased in brain-injured rats. This is in agreement with previous studies demonstrating that injury of the spinal cord (Wang *et al*., 2002; Hunt *et al*., 2003) or the brain (Meier *et al*., 2003; Mingorance *et al*., 2004; Marklund *et al*., 2006; Thompson *et al*., 2006) increases expression of myelin growth inhibitors. FPI has been shown to increase MAG and Nogo-A in the hippocampus where these two molecules function as normal components of myelin-ensheathing axonal tracts (Trapp *et al*., 1989; Huber *et al*., 2002). Axonal elements are preferentially vulnerable to the effects of TBI (Pettus *et al*., 1994; Povlishock & Christman, 1995). It is possible that elevated MAG and Nogo-A may retard the postinjury regenerative attempts to repair damaged axonal circuitry. Additionally, increased expression of Nogo-A after injury may also contribute to alterations of neuronal function as Nogo-A is expressed in neurons as well (Wang *et al*., 2002; Chaudhry & Filbin, 2007; Zhao *et al*., 2007). Importantly, MAG and Nogo-A were selectively up-regulated after FPI without changing the total level of hippocampal myelin, as injury did not affect levels of MBP, one of the major proteins of CNS myelin (Quarles, 2002).

We measured levels of GAP-43 and SYP, which are normally found in growing axon terminals and membranes of presynaptic vesicles, to obtain an estimate for the effects of FPI on axonal growth and synapse formation and/or adaptation. The results showing that changes in levels of MAG and Nogo-A were associated with opposite changes in GAP-43 and SYP after trauma may reflect an action of MAG and Nogo-A on reducing the capacity for synaptic plasticity and neurite outgrowth. CNS injury is generally followed by a period of axonal sprouting, which has been associated with up-regulation of GAP-43 and SYP (Christman *et al*., 1997; Emery *et al*., 2000; Shojo & Kibayashi, 2006). However, this regenerative response is generally short-lasting and varies depending on the type and severity of the injury (Hulsebosch *et al*., 1998; Thompson *et al*., 2006). Our results showing reduced expression of GAP-43 and SYP after mild FPI could reflect their overall decline over time following an initial regenerative attempt. Elevated levels of MAG and Nogo-A may increase the growth-inhibitory substrate of the brain by reducing GAP-43 and SYP levels and thus counteract the postinjury reparative efforts.

It is possible that the treatment strategies to reduce expression of MAG and Nogo-A contribute to overcoming the learning deficits observed after TBI (Wu *et al*., 2004). Deficits in memory and information processing are detrimental features of TBI (Levin, 1998; Wu *et al*., 2004). Behavioural recovery after TBI may be facilitated by regulating levels of myelin growth inhibitors (Buchli & Schwab, 2005; Thompson *et al*., 2006). For example, the application of MAG (Thompson *et al*., 2006)- or Nogo-A (Lenzlinger *et al*., 2005)-neutralizing antibodies improves cognitive function in animals exposed to brain injury.

Exercise modulated myelin growth-inhibitory molecules via a mechanism involving BDNF and PKA

We then sought to determine whether voluntary exercise may help counteract the inhibitory effects of MAG and Nogo-A on axonal and synaptic integrity observed after CNS injury (Grandpre *et al*., 2000; Domeniconi & Filbin, 2005; Mimura *et al*., 2006; Chaudhry & Filbin, 2007). The application of voluntary exercise following TBI significantly reduced MAG and Nogo-A levels and increased GAP-43 and SYP in both sham-injured and brain-injured rats. The increasing running distance was correlated with decreasing levels of MAG and Nogo-A and increasing levels of GAP-43 and SYP. Importantly, these effects of exercise were abolished following BDNF blockade, suggesting an involvement of BDNF in the modulation of growth inhibitors and growth-promoting molecules by exercise. These results are supported by several *in vitro* studies demonstrating that neurons primed with BDNF become resistant to the inhibitory action of MAG (Cai *et al*., 1999; Gao *et al*., 2003; Spencer & Filbin, 2004). We have also previously shown that voluntary exercise enhances expression of GAP-43 and SYP in the adult spinal cord and hippocampus (Gomez-Pinilla *et al*., 2002; Vaynman *et al*., 2006) and that BDNF blockade prevents the exercise-induced increase in SYP (Vaynman *et al*., 2006).

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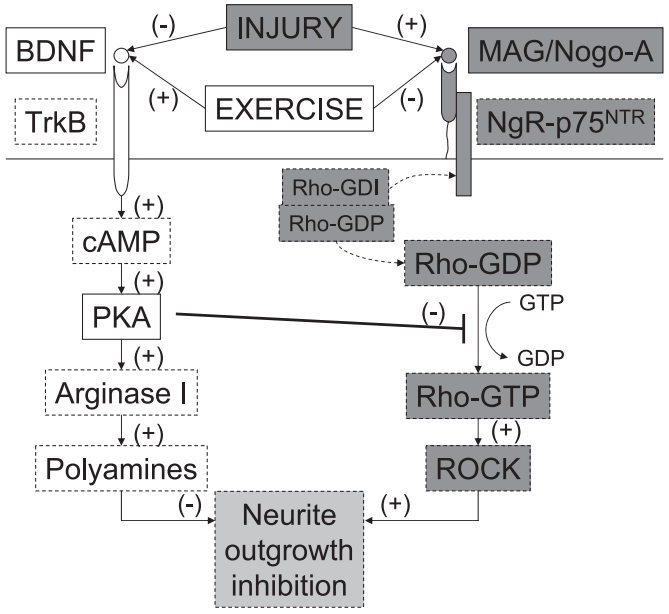

FIG. 5. Schematic diagram depicting the effects of injury and exercise on the key molecular components involved in the mechanism of neurite outgrowth inhibition triggered following CNS injury. Interaction of MAG or Nogo-A with a common receptor complex, NgR-p75^{NTR}, displaces small GTPase RhoA from Rho guanine dissociation inhibitor (Rho-GDI), allowing activation of RhoA and subsequently its key downstream effector, RhoA-associated kinase (ROCK), leading to inhibition of neurite outgrowth. PKA levels are modulated by injury and/or exercise in a similar manner as BDNF. The interaction of BDNF with TrkB receptors induces cAMP-dependent activation of PKA, which can block a signalling pathway activated by MAG and Nogo-A through inactivation of RhoA and/or induce arginase 1-dependent synthesis of polyamines to block the effects of MAG and Nogo-A. We propose that voluntary exercise can overcome the effects of injury on myelin growth-inhibitory molecules by activating the BDNF system. The components of the diagram describing the present findings are shown in boxes with a full line and the components from existing literature are shown in boxes with a dashed line.

Additionally, SYP has been reported to be significantly reduced in hippocampal synaptosomes of BDNF-knockout mice (Pozzo-Miller *et al*., 1999).

Neural activity associated with exercise may be a driving factor for the changes in myelin growth-inhibitory molecules observed in this study. This notion is supported by findings that a brief electrical stimulus can switch the turning response of a growing axon to MAG from repulsion to attraction (Ming *et al*., 2001; Henley *et al*., 2004). Neural activity regulates the induction and release of BDNF (Goodman *et al*., 1996; Poo, 2001; Lessmann *et al*., 2003), and exercise also modulates BDNF expression in neurons and astrocytes (Gomez-Pinilla *et al*., 2002; Vaynman *et al*., 2004). Additionally, the expression of TrkB receptors is up-regulated in neurons and oligodendrocytes following long-term treadmill walking (Skup *et al*., 2002). Therefore, BDNF/TrkB signalling enhanced after exercise in either neurons or glial cells may contribute to the autocrine and/or paracrine regulation of MAG and Nogo-A (Dougherty *et al*., 2000; Dai *et al*., 2001; Skup *et al*., 2002).

Activation of PKA is involved in the process by which BDNF overcomes myelin inhibition (Cai *et al*., 1999; Gao *et al*., 2003). Our results show that exercise increased PKA levels and this effect was abolished following BDNF blockade. Activated PKA can trigger nuclear transcription of arginase 1 enzyme, which leads to the synthesis of polyamines to overcome the inhibition by myelin growth inhibitors (Cai *et al*., 2002; Chaudhry & Filbin, 2007; Fig. 5). Additionally, activation of PKA blocks the conformation of the small GTPase RhoA into its active GTP-bound form (Lang *et al*., 1996:; Laudanna *et al*., 1997), a critical step in the inhibitory signal transduction by MAG and Nogo-A. RhoA is activated by MAG and Nogo-A as their main intracellular effector, following the release of RhoA from its suppressor, Rho-GDI (Yamashita & Tohyama, 2003), with the subsequent activation of RhoA-associated kinase (Govek *et al*., 2005) to induce arrest of axonal growth and neurite retraction (Fig. 5). Therefore, termination of RhoA signalling can promote axon regeneration (Lehmann *et al*., 1999), possibly as a result of exercise-induced up-regulation of BDNF and PKA. We also found that TBI decreased PKA levels, which may in turn enhance RhoA activation by MAG and Nogo-A elevated after injury. This notion is supported by recent reports showing that TBI significantly increases activation of RhoA in the rat hippocampus (Dubreuil *et al*., 2006) as well as in human brains (Brabeck *et al*., 2004).

Therapeutic implications for the effects of exercise on growth inhibitors

Using exercise as a physiological treatment strategy in combination with pharmacological and/or genetic approaches may expedite the healing process after TBI. Studies have shown pharmacological and genetic agents to be effective in modulating the action of myelin growth inhibitors (David & Lacroix, 2003; Schwab, 2004; Domeniconi & Filbin, 2005). Applications of MAG- or Nogo-A-neutralizing antibodies following injury increase the rate of regeneration (Buchli & Schwab, 2005; Teng & Tang, 2005; Schweigreiter & Bandtlow, 2006) and anti-MAG antibody treatment can also reduce injury-related tissue loss (Thompson *et al*., 2006). Additionally, genetic deletions of MAG (Bartsch *et al*., 1995), Nogo-A (Zheng *et al*., 2003) or their receptor components (Song *et al*., 2004; Zheng *et al*., 2005) have demonstrated a degree of success in inducing regeneration after injury. However, the fact that myelin growth inhibitors interact with a common receptor complex, NgR-p75^{NTR} (McGee & Strittmatter, 2003; Spencer *et al*., 2003; Yiu & He, 2003), makes targeting MAG and Nogo-A challenging for counteracting the inhibitory action of myelin. The redundancy of myelin-derived inhibitory pathways may thus limit the potential of select pharmacological agents for regeneration. Accordingly, the physiological nature of exercise has the advantage of promoting changes in a comprehensive set of molecular pathways that are required for normal function. For example, the potential of exercise to reduce the action of myelin growth inhibitors may supplement its capacity to directly promote axonal and synaptic growth. We have also recently found that exercise can modulate expression of MAG in the spinal cord (Ghiani *et al*., 2007), possibly via a similar mechanism as in the brain. Future studies should therefore focus on developing such strategies that can reduce the growth-inhibitory and increase the growth-promoting substrate of the brain to stimulate brain plasticity after TBI.

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Abbreviations

BDNF, brain-derived neurotrophic factor; BSA, bovine serum albumin; CNS, central nervous system; FPI, fluid percussion injury; GAP-43, growth associated protein 43; MAG, myelin-associated glycoprotein; MBP, myelin basic protein; PBS, phosphate-buffered saline; PKA, protein kinase A; SYP, synaptophysin; TBI, traumatic brain injury.

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