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Exercise-Induced Behavioral Recovery and Neuroplasticity in the 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine-Lesioned Mouse Basal Ganglia

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Physical activity has been shown to be neuroprotective in lesions affecting the basal ganglia. Using a treadmill exercise paradigm, we investigated the effect of exercise on neurorestoration. The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned mouse model provides a means to investigate the effect of exercise on neurorestoration because 30–40% of nigrostriatal dopaminergic neurons survive MPTP lesioning and may provide a template for neurorestoration to occur. MPTP-lesioned C57 BL/6J mice were administered MPTP (four injections of 20 mg/kg free-base, 2 hr apart) or saline and divided into the following groups: (1) saline; (2) saline + exercise; (3) MPTP; and (4) MPTP + exercise. Mice in exercise groups were run on a motorized treadmill for 30 days starting 4 days after MPTP lesioning (a period after which MPTP-induced cell death is complete). Initially, MPTP-lesioned + exercise mice ran at slower speeds for a shorter amount of time compared to saline + exercise mice. Both velocity and endurance improved in the MPTP + exercise group to near normal levels over the 30-day exercise period. The expression of proteins and genes involved in basal ganglia function including the dopamine transporter (DAT), tyrosine hydroxylase (TH), and the dopamine D1 and D2 receptors, as well as alterations on glutamate immunolabeling were determined. Exercise resulted in a significant downregulation of striatal DAT in the MPTP + exercise compared to MPTP nonexercised mice and to a lesser extent in the saline + exercised mice compared to their no-exercise counterparts. There was no significant difference in TH protein levels between MPTP and MPTP + exercise groups at the end of the study. The expression of striatal dopamine D1 and D2 receptor mRNA transcript was suppressed in the saline + exercise group; however, dopamine D2 transcript expression was increased in the MPTP + exercise mice. Immunoelectron microscopy indicated that treadmill exercise reversed the lesioned-induced increase in nerve terminal glutamate immunolabeling seen after MPTP administration. Our data demonstrates that exercise promotes behavioral recovery in the injured brain by modulating genes and proteins important to basal ganglia function.
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Key words: tyrosine hydroxylase; dopamine transporter; dopamine receptors; glutamate; MPTP; Parkinson's disease

It has been established that neuroplasticity, as characterized by neurogenesis, synaptogenesis, and molecular adaptations, exists in the human nervous system. Animal models of brain injury have provided a means to both investigate and manipulate neuroplasticity. A heightened area of interest is the role that exercise plays in facilitating neuroplasticity in either the noninjured or injured brain (Fisher et al., 2001). Studies employing a variety of animal models of injury have shown that exercise can promote neuroplasticity and behavioral recovery in the hippocampus, cortex, and spinal cord (Kempermann et al., 2000). In

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rodent models of basal ganglia injury, exercise has been shown to be neuroprotective (Tillerson et al., 2001, 2003; Tillerson and Miller, 2002). By restraining the unimpaired limb immediately after injury and forcing use of the impaired upper limb, behavioral and neurochemical sparing were demonstrated in the 6-hydroxydopamine (6-OHDA)-lesioned rat. This suggested that forced use of the impaired limb protected dopaminergic cells from the neurotoxic effects of 6-OHDA. In addition, Tillerson et al. (2003) reported behavioral improvement after treadmill exercise in two rodent models of basal ganglia injury (the 6-OHDA rat and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [MPTP]-lesioned mouse) (Tillerson et al., 2003). Immediate exposure to treadmill training within 12 hr of injury was associated with attenuation of dopamine loss. The investigators concluded that exercise might work largely through neuroprotective mechanisms because exercise was started within 12 hr of lesioning, and MPTP and 6-OHDA may take several days to complete cell death (Sauer and Oertel, 1994; Jackson-Lewis et al., 1995). The implication of these neuroprotective studies is that exercise may be helpful in delaying or preventing Parkinson's disease in healthy individuals (Sasco et al., 1992).

The interest in our laboratory is to investigate the role of exercise in promoting repair of the injured basal ganglia. We define this as neurorestoration, which is the capacity of surviving dopaminergic neurons to adapt after injury with potential behavioral benefits. The MPTP-lesioned mouse model of basal ganglia injury provides a means to investigate neurorestoration because 30–40% of the substantia nigra pars compacta (SNpc) dopaminergic neurons survive the lesioning regimen. Despite a 90% loss of striatal dopamine, these mice display robust and reproducible return of striatal dopamine, tyrosine hydroxylase (TH) protein, and dopamine transporter (DAT) protein 2–3 months after MPTP lesioning. This suggests that surviving nigrostriatal dopaminergic neurons provide a template for neurorestoration and would therefore provide a means to investigate the effect of exercise on facilitating neurorestoration. In addition, MPTP-lesioned mice manifest motor behavioral deficits that can be monitored throughout the recovery process (Tillerson et al., 2003). The purpose of our study was to investigate the effect of treadmill exercise on neurorestoration using the MPTP-lesioned mouse model of basal ganglia injury by introducing exercise 4 days after MPTP lesioning, a time point well after cell death is complete. Because TH, DAT, dopamine (D1 and D2) receptors and glutamate storage have been shown to be altered in the MPTP-lesioned mouse model (Jakowec et al., 2004), we chose to examine these same parameters in our MPTP exercise paradigm. Exercise was continued for 30 days to parallel the 30-day recovery period when TH is beginning to return.

MATERIALS AND METHODS

Housing and Acclimation of Mice

Young adult (8–10 weeks old) male C57BL/6J mice supplied from Jackson Laboratory (Bar Harbor, ME) were used for this study. There were four treatment groups: (1) saline injected; (2) saline + exercise; (3) MPTP lesioned; and (4) MPTP lesioned + exercise. Three cohorts of mice, consisting of four groups of 10 mice/group were used (total n + 120 mice). Animals were housed six to a cage and acclimated to a 12-hr shift in light/dark cycle so that exercise occurred during the animals' normal wake period.

MPTP Lesioning

MPTP (Sigma, St. Louis, MO) was administered in a series of four intraperitoneal injections of 20 mg/kg (free-base) at 2-hr intervals for a total administration of 80 mg/kg. This regimen leads to a 60–70% loss of nigrostriatal neurons (as determined by unbiased stereologic techniques for both TH staining and Nissl substance in our laboratory) and an 80–90% depletion of striatal dopamine levels (Jackson-Lewis et al., 1995). Using this regimen, nigrostriatal cell loss is complete by Day 3 after MPTP administration and persists beyond 30 days post-lesioning as determined by either unbiased stereologic counting techniques (Petzinger et al., in preparation) or section-sampling techniques (Jackson-Lewis et al., 1995).

Selection of Mice and Exercise Protocol

Figure 1A outlines the experimental design of animal groups. Before MPTP lesioning, a baseline treadmill running assessment was conducted to insure that all animals performed similarly on the treadmill task before MPTP lesioning. Forty animals that could maintain a forward position on the 2.5-m treadmill belt for 5 min at 10 m/min were assigned randomly to the four groups. A non-noxious stimulus (metal-beaded curtain) was used as a tactile incentive to prevent animals from drifting back on the treadmill. Consequently, shock-plate incentive was not used and stress related to the activity was minimized. Exercise was initiated 4 days after saline or MPTP lesioning. For exercise training, a motorized, 10-lane rodent treadmill (Fig. 1B) was used at incremental speeds to a goal treadmill speed between 20.5–23.0 m/min (Fukai et al., 2000). All 10 mice from each of the two exercise groups (MPTP + exercise and saline + exercise) were run at the same time in the 10-lane treadmill (see Fig. 1B). Exercise duration was incrementally increased to reach the goal duration of 2 × 30 min/day (60 min), 5 days/week (with a 2-min warmup period) for a total of 30 days of exercise. Treadmill speed for each group was increased when all 10 mice within each group maintained a forward position on the 2.5-m treadmill belt for 75% of the running period. When all 10 mice within each of the two running groups maintained a forward position 100% of the time, duration for that group was increased. Mice were exercised as a group of 10 for two 30-min sessions (total 1 hr) per day with a 30-min rest period between sessions. To control for any non-exercise effects of treadmill running (handling, novel environment, noise, and vibration) nonexercised groups were placed on the top of the treadmill apparatus for a time period equivalent to

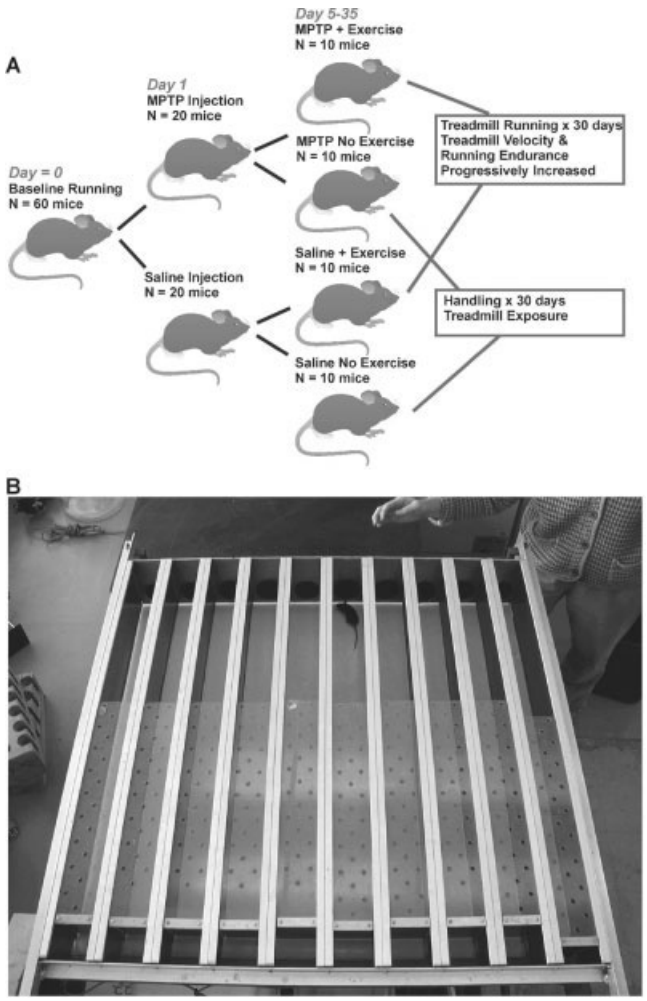

Fig. 1. **A:** Summary of experimental design with group assignment and exercise protocol. **B:** The 10-lane motorized treadmill used for exercising the mice.

exercise training (Fukai et al., 2000; Kojda et al., 2001). At the end of the 30-day running period, all animals from the four groups (exercise and non-exercise, with and without MPTP) were run to compare running speed capability. Initial treadmill velocity was set at the same speed at which initial pre-exercise baseline running capability was determined (i.e., 10 m/min). Maximum velocity for each group was defined as the velocity at which the mice, as a group of 10, could maintain a forward position on the treadmill for 75% of a 5-min running trial.

Collection of Brain Tissue

Brain tissue was collected at 4 days post-MPTP lesioning to examine the reduction in the degree of DAT and TH immunoreactivity. Brain tissue was collected from all groups at the conclusion of the 30 days of treadmill exercise, (35 days post-MPTP lesioning). Tissue for immunohistochemical analysis was fixed by transcardial perfusion with 50 ml of ice-cold saline followed by 50 ml of 4% paraformaldehyde/phosphate-buffered saline (PFA/PBS) pH 7.2. Brains were removed, post-fixed in 4% PFA/PBS for 48 hr, cryoprotected in 20% sucrose for 24 hr, and then quickly frozen in isopentane on dry ice. Tissues for Western immunoblotting and in situ hybridization were harvested fresh after cervical dislocation. All procedures used in these studies adhered to the guidelines of the Institutional Animal Care and Use Committee (IACUC) and the National Institutes of Health. To maximize the utilization of brain tissues each technique involving either fresh tissues (for Western immunoblotting), quick frozen tissues (for in situ hybridization histochemistry), or perfusion fixed (for immunoelectron microscopy or immunohistochemistry) consisted of at least 6 to as many as 10 mice from each group in a single experimental cohort. This assured a large enough *n* to detect changes within each experimental design.

Immunohistochemistry

Fixed tissue from at least six mice from each group was cut at 30-μm thickness, placed in phosphate buffer, and used immediately for immunohistochemistry. Commercially available antibodies included rabbit polyclonal anti-TH (Chemicon, Temecula, CA), and mouse monoclonal anti-DAT (Chemicon). Tissue sections were washed in Tris-buffered saline (TBS; 50 mM Tris pH 7.4 and 0.9% NaCl) and exposed to antibody (1:1,000) for 48 hr at 4°C. Sections were washed in TBS, and exposed to horseradish peroxidase (HRP)-conjugated secondary antibody using the ABC Elite kit (Vector Labs, Burlingame, CA). Antibody staining was visualized by development in DAB/H_2O_2. To ensure that differences in staining intensity were due to differences in antigen expression, multiple sections from each of the different treatment groups were handled concurrently in identical staining conditions. Control experiments excluding either primary antibody or secondary antibody were also carried out to verify staining specificity. Determination of the relative expression of TH immunoreactivity (ir) and DAT-ir in the striatum using immunohistochemistry from different mouse groups was based on published validity studies (Burke et al., 1990). For image analysis, three or four animals per treatment group and 10–12 sections per animal, spanning the midstriatum rostral to the anterior commissure (Bregma 0.25–1.25 mm) were used. Striatal images were captured at low magnification and digitized. The relative optical density (OD) (expressed as arbitrary units within the linear range of detection) of the dorsal lateral striatum was determined by subtracting the relative optical density of the corpus callosum as background. To ensure that the gray values represented an OD within the nonsaturated range of the image analysis, a Kodak Photographic step tablet (density rage to 255 OD units) captured by the CCD camera was used. Maximal tissue immunostaining relative OD units did not exceed the relative OD units of the tablet.

Western Immunoblotting

Tissue for Western blot analysis was dissected from the mid-striatum (a 3-mm thick section between Bregma 0.00–1.50) of at least six mice from each group and homogenized in buffer (25 mM Tris pH 7.4, 1 mM EDTA, 100 μM phenylmethylsulfonylfluoride [PMSF]). Protein concentration was determined by the BCA method (Pierce, Inc.). Proteins (10 μg) were

separated by polyacrylamide gel electrophoresis (PAGE) using the method of Laemmli (1970) and transferred to nitrocellulose filters by electroblotting in Towbin buffer (Towbin et al., 1979). Filters were blocked in TS-Blotto (50 mM Tris pH 7.4, 0.9% NaCl, 5% nonfat milk) for 1 hr, then exposed to primary antibody (1:2,000) in TS for 2 hr. Filters were then washed in TS, and exposed to secondary antibody in TS-Blotto for 1 hour. After a final wash in TS, antibody binding was visualized by chemiluminescence (Pierce, Inc.) and apposing filters to film (Hyperfilm ECL; Amersham) and processed in X-OMAT developer. Images were scanned and the relative OD of bands (expressed as arbitrary units within the linear range of film) was determined using Bioquant Nova Prime, a computer-assisted image analysis program (Bioquant Imaging, Nashville, TN).

Electron Microscopy/Immunocytochemistry

Electron microscopic immunolabeling for glutamate was carried out on mice from the saline group (n + 6), saline + exercise group (n + 10), MPTP group (n + 7), and MPTP + exercise group (n + 9). Anesthetized mice were perfused transcardially with 6 ml of heparin (1,000 U/ml) in HEPES buffer (pH 7.3) followed by 50 ml of 2.5% glutaraldehyde/0.5% paraformaldehyde in HEPES (pH 7.3) containing 0.1% picric acid. The brain was removed and post-fixed overnight at 4°C. Vibratome sections (200-mm thick) were cut in the coronal plane through the striatum and the dorsal hippocampus. A 2 × 2 mm^2 piece of the dorsolateral striatum (site of the major input of the corticostriatal pathway) and the CA1 subregion of the hippocampus (used as a control area to look for nonspecific effects of exercise), were dissected, washed in HEPES buffer, incubated at room temperature in the dark in aqueous 1% osmium tetroxide/1.5% potassium ferricyanide, washed in deionized water and en block stained with aqueous 0.5% uranyl acetate at room temperature for 30 min. The tissue was dehydrated, embedded in Embed 812/Spurr's (EMS; Fort Washington, PA) and sections were cut and stained. Post-embedding immunogold electron microscopy was carried out according to a modified method of Phend (Phend et al., 1992; Tillerson et al., 2003). Thin sections (light gold interface color) were cut and placed on 200-mesh nickel coated grids double coated previously with a solution from a Coat-Quick "G" pen (Kiyota International, Elk Grove, IL.), air dried for several hours, and washed for 5 min in TBS with Triton X-100 (TBST; 0.05 M Tris, pH 7.6, 0.9% NaCl, and 0.1% Triton X-100). The grids were transferred to the primary antibody solution and incubated overnight in a moist chamber. The glutamate antibody (non-affinity purified, rabbit polyclonal; Sigma, St. Louis, MO), as characterized previously by Hepler et al. (1988), was diluted 1:400,000 in TBST 7.6. Aspartate (1 mM) was added to the glutamate antibody mixture 24 hr before incubation with the thin-sectioned tissue to prevent any cross-reactivity with aspartate within the tissue. The grids were incubated for 1.5 hr at room temperature in goat anti-rabbit IgG conjugated to 10-nanometer gold (diluted 1:50 in TBST 8.2; Amersham). Photographs (10/animal) were taken randomly throughout the section containing the caudate nucleus or the CA1 region of the hippocampus (1 section/grid, one photograph per grid square) at a final magnification of 40,000× within the area of the neuropil (location of the greatest number of synapses) by an individual blinded to the particular experimental group and then captured on the computer using an AMT (2K × 2K) digital camera (Danvers, MA). The number of gold particles per nerve terminal associated with an asymmetrical (glutamate) synaptic contact and the area of the nerve terminal was determined using Image Pro Plus imaging software (Media Cybernetics, Tacoma, WA). The gold particles contacting the synaptic vesicles within the nerve terminal were counted and considered part of the vesicular or neurotransmitter pool by previously determined methods (Meshul et al., 1998; Tillerson et al., 2003). The specificity of immunolabeling for the glutamate antibody was established by incubating the antibody overnight with 3 mM glutamate. Preabsorption of the glutamate antibody resulted in a total lack of tissue labeling. The density of glutamate immunolabeling within the mitochondria associated with the presynaptic terminal was determined also as a means of investigating changes in the presynaptic metabolic pool of glutamate. The mean density of gold particles/μm^2 ± SEM) was determined within each treatment group. Stereologic analysis was not carried out for the nerve terminal glutamate immunolabeling study, because synapse density was not being determined, only the density of gold particles per identified nerve terminal making an asymmetrical synaptic contact.

In Situ Hybridization

Brains for in situ hybridization were removed quickly and frozen in isopentane on dry ice. Sections were cut 14-μm thick on a Jung 1850 cryostat (Leica, Inc.) and thaw mounted onto poly-L-lysine-coated microscope slides, dried on a 55°C slide warmer, and fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS), pH 7.2. Sections were dehydrated in successive ethanol washes (30, 60, 80, 95, and 100%), deprotonated in triethanolamine/acetic anhydride, delipidated in chloroform, and dehydrated in ethanol. Slides containing tissue sections were exposed to hybridization buffer containing 4× standard sodium citrate (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 50% formamide, 1× Denhardt's Solution, 250 μg/ml tRNA, 500 μg/ml salmon sperm DNA, and 10% dextran sulfate with 1 × 10^6 cpm of probe. Sections were cover-slipped and incubated overnight at 44°C. Tissue sections labeled with ribonucleotide probes were washed first in 2× SSC/50% formamide/0.1% β-mercaptoethanol at 37°C for 30 min, then 20 μg/ml RNase in 0.5 M NaCl/10 mM Tris pH7.4 at 37°C for 30 min, followed by 2× SSC/50% formamide/0.1% β-mercaptoethanol at 60°C for 1 hr, 0.1× SSC/0.1% β-mercaptoethanol at 65°C for 1 hour, and finally rinsed in ethanol before air drying. Slides were placed against high-resolution film (Hyperfilm B-max; Amersham) with radioactive standards (Amersham, Inc.). Selected slides were dipped in NTB-2 (Kodak) photographic emulsion, developed in D-19 developer and counter stained with eosin. To minimize potential sources of variation between different experiments, slides that were to be compared were processed in the same experiment using identical hybridization cocktail, probe concentration, probe preparation, wash regimen, and film exposure. The computerized image analysis program Bioquant was used to

determine the number of emulsion grains above specific anatomic regions within the substantia nigra pars compacta.

Statistical Analysis

Linear regression was carried out to compare the rate of change in velocity and endurance of treadmill running between the two groups. Treatment groups were compared using one-way analysis of variance (ANOVA), followed by the Fisher post hoc test for comparison of multiple means for the following measures: DAT, TH, dopamine D1 and D2 receptors, and glutamate immunogold labeling. All analyses were carried out with SPSS software. Statistical significance was accepted at $P < 0.05$.

RESULTS

Exercise-Induced Changes in Behavior

Figure 1 outlines the four animal groups used in this study. Two groups (saline + exercise and MPTP + exercise) were subjected to treadmill exercise for 30 days and changes in running duration and velocity were measured. Duration increased over the 30-day exercise period for both groups (see Fig. 2A). Both the saline + exercise and MPTP + exercise groups were capable initially of running for a duration of 30 min on Day 1, which increased to a maximal duration of 60 min. The saline + exercise group reached maximal duration by Day 12, however, whereas the MPTP + exercise group did not reach maximal duration until Day 26. As such, we compared the rate of increase in exercise duration over the first 12 days of running. The rate of change of duration for the saline + exercise group was significantly greater than that seen in the MPTP + exercise group ($P < 0.05$).

Similar to duration, running velocity increased in both the saline + exercise and MPTP + exercise groups over the 30-day period (see Fig. 2B). The saline group ran at a velocity of 13.3 m/min at Day 1 and increased to 23.0 m/min by Day 30. The MPTP group ran at a velocity of 6.3 m/min at day 1 and increased to 21.7 m/min by day 30. There was a significant difference in velocity at Day 1 between the two groups (saline + exercise, 13.3 m/min; MPTP + exercise

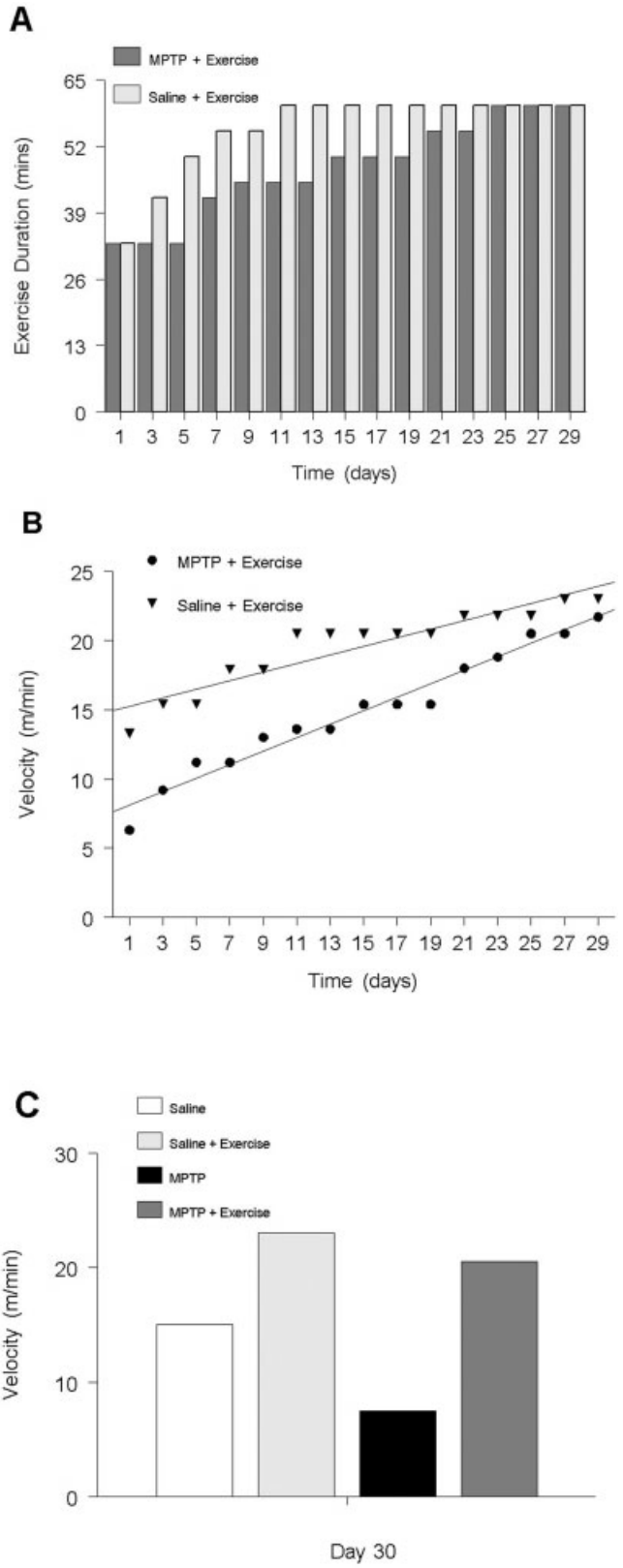

Fig. 2. Exercise-induced changes in behavior. **A:** Change in running duration over the 30-day running period for the saline + exercise group (gray bars) and MPTP + exercise group (black bars). The bars represent the performance of all 10 mice/group running at the same time. The increase over days of running reflects that all 10 mice met the criteria for increasing running duration. No statistical analysis was carried out because each bar represents all 10 mice/group as a single data point. **B:** Change in running velocity (in m/min) over the 30-day running period for the saline + exercise group (triangles) and MPTP + exercise group (circles). Symbols represent the performance of all 10 mice in each running group; increase over days of running reflects that all 10 mice met the criteria for increasing running velocity. **C:** Compares running velocity between the four groups (saline, white bar; MPTP, light gray bar; saline + exercise, black bar; and MPTP + exercise, dark gray bar) at the conclusion of the running program on Day 30. The bars represent performance of all 10 mice/group from the four groups running at the same time.

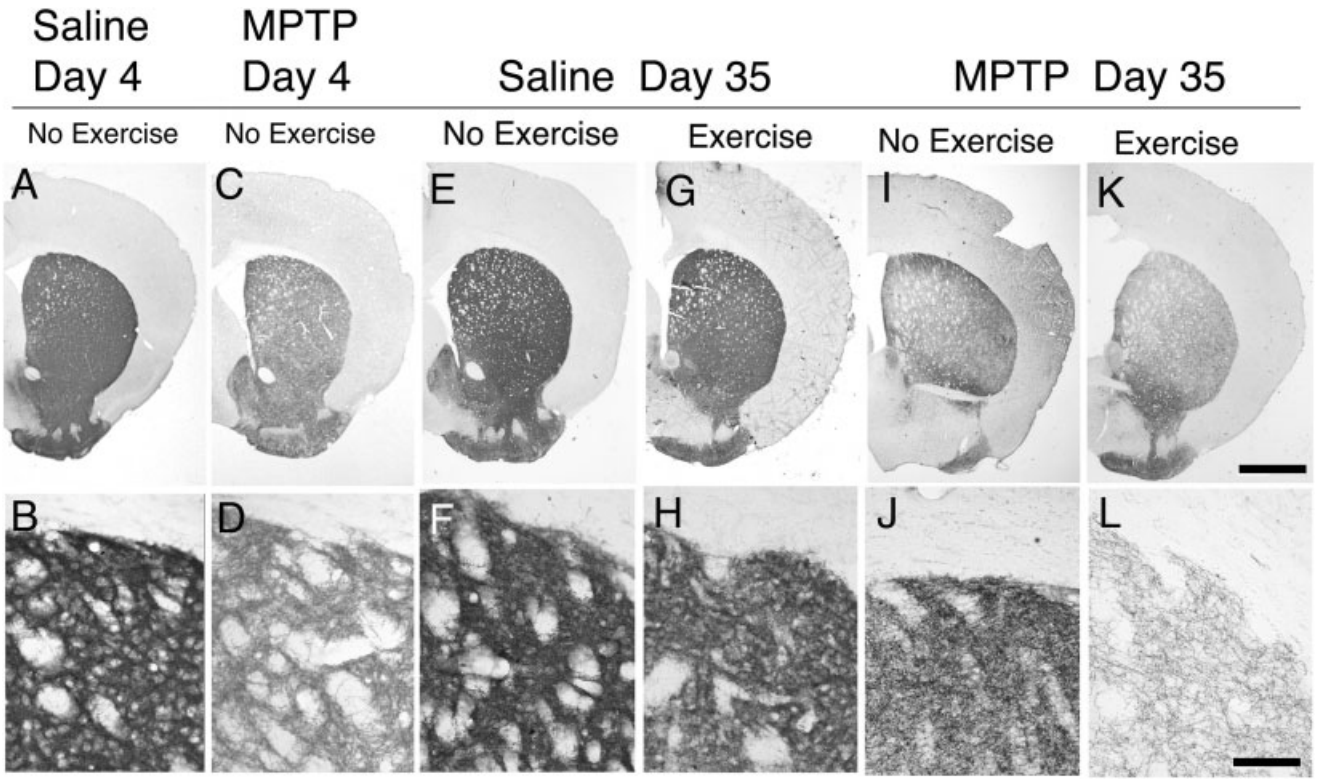

Fig. 3. Exercise-induced changes in striatal dopamine transporter immunolabeling. The relative expression of striatal dopamine transporter protein (DAT) after MPTP lesioning and between different treatment groups was determined using immunohistochemistry. The upper panels show low-magnification (10×) images of coronal sections at the level of the midstriatum stained with an antibody against DAT protein. The lower panels show high-magnification (400×) images from a region corresponding to the dorsal lateral striatum from respective sections in the upper panels. MPTP lesioning leads to a reduction in DAT-ir (comparing saline in **A** and **B** and MPTP in **C** and **D** at Day 4). MPTP + exercise showed reduced DAT-ir (**K** and **L**) compared to MPTP without exercise (**I** and **J**). Saline + exercise (**G** and **H**) showed a slight reduction in DAT-ir compared to saline alone (**E** and **F**). Scale bar + 0.5 mm (in K, for upper panels); 50 μm (in H, for lower panels).

group, 6.3 m/min; 7.0 m/min difference between groups at Day 1; $P < 0.0001$). This difference reduced to 1.3 m/min by Day 30 (Fig. 2B). The change in velocity over days of running resulted in a significantly different rate of change between the two groups ($P < 0.0001$). The MPTP + exercise group increased treadmill velocity by 5 m/min per day compared to 3.1 m/min per day for the saline + exercise group (Fig. 2B).

At the end of the 30-day running period, all animals from the four groups (saline, saline + exercise, MPTP, and MPTP + exercise) were tested on the treadmill to compare running speed capability (Fig. 2C). The MPTP and MPTP + exercise groups had a treadmill velocity of 7.5 and 21.7 m/min, respectively. Interestingly, the running velocity of the MPTP group at 35 days post-MPTP lesioning (7.5 m/min) was similar to the MPTP + exercise group at Day 1 (6.3 m/min) of their treadmill exercise program. Taken together, these findings indicate that there was no spontaneous increase in running velocity in the MPTP nonexercised group. The intensity of our training regimen was substantiated by the fact that there was an effect of exercise in the saline + exercise group compared to the saline nonexercised group. The comparison of running speed capability (Fig. 2C) at the end of the exercise program demonstrated differences in running velocity between the saline groups (saline + exercise, 23 m/min; saline nonexercised, 15 m/min).

Exercise-Induced Changes in Striatal Dopamine Transporter Protein

Analysis of the pattern of expression of DAT protein in the midstriatum (Bregma level +1.00) in both the saline (Fig. 3A,B) and MPTP groups (Fig. 3C,D) at Day 4 showed a significant reduction in DAT-ir due to MPTP lesioning. After the exercise regimen (Day 35 post-lesioning) the saline group showed the highest degree of striatal DAT-immunoreactivity (DAT-ir) compared to that in all other groups (Fig. 3E,F). Interestingly, the saline + exercise group had reduced DAT-ir compared to the saline group, which suggests that exercise itself reduced DAT-ir (compare Fig. 3E,F with Fig. 3G,H). This reduction was shown to be significant (saline, 100.0 ± 3.1%; saline + exercise, 84.3 ± 2.8%; $P < 0.006$) (see Fig. 4). In addition, the MPTP + exercise group demonstrated sig-

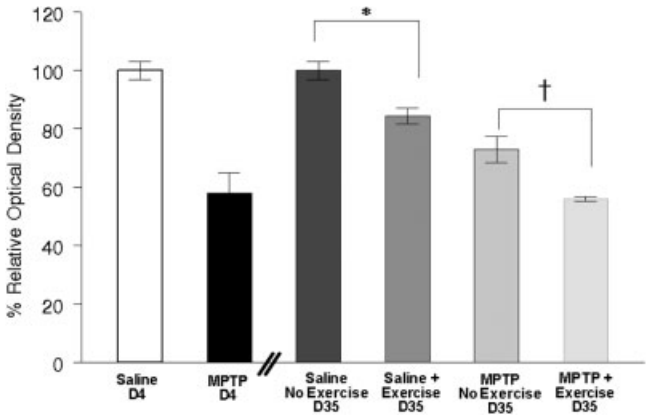

Fig. 4. Analysis of exercise-induced expression of dopamine transporter immunolabeling: The relative striatal DAT-ir was determined by measuring the relative optical density of the dorsal lateral quadrant (in at least 12 sections) correcting for staining background within sections by measuring the relative OD of the corpus callosum. This data represents relative staining for representative sections from the saline and MPTP groups at Day 4 post-MPTP lesioning and the four treatment groups collected after completion of the exercise program at post-MPTP lesioning Day 35. For comparisons, the Day 4 saline group was arbitrarily set as 100% (mean ± SEM, 100 ± 3.1%) and the relative optical densities of all other groups were normalized against it. Comparison of DAT-ir at Day 4 post-MPTP lesioning (58.0 ± 7.0%) showed a significant reduction in DAT-ir. The asterisks and cross indicate statistically significant differences between the saline and saline + exercise and the MPTP and MPTP + exercise groups, respectively. This reduction was significant (saline, 100.0 ± 3.1%; saline + exercise, 84.3 ± 2.8%; $P < 0.006$). In addition, the MPTP + exercise group demonstrated significantly reduced DAT-ir compared to the MPTP group (MPTP, 73.3 ± 4.5%; MPTP + exercise, 56.0 ± 0.7%; $P < 0.028$).

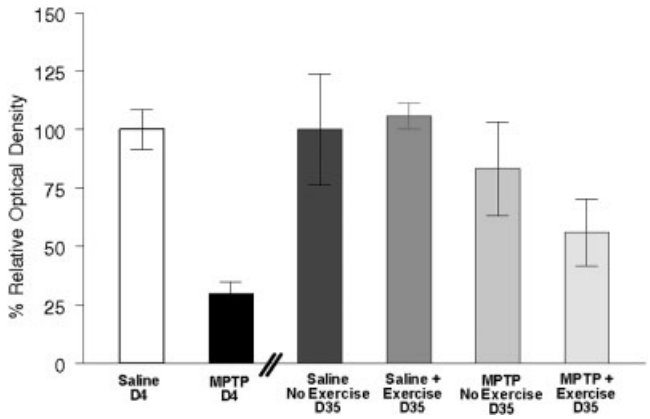

Fig. 5. Analysis of the relative striatal TH-protein expression by Western immunoblotting. The relative optical density measurement of the saline group at Day 4 post-lesioning was set arbitrarily at 100 ± 3.1% for comparison between different treatment groups. Analysis of TH-protein levels at 4 days after MPTP lesioning showed a significant reduction (30.0 ± 4.5%) compared to that in saline controls. There were no significant differences between groups in TH-ir at 35 days post-MPTP lesioning (saline, 100.0 ± 23.8%; saline + exercise, 105.8 ± 5.7%; MPTP, 83.3 ± 20.0%; MPTP + exercise, 56.0 ± 14.3%; $P + 3.72$). These data, however, indicate a small, nonsignificant reduction in the relative density of TH-ir in the MPTP groups compared to that in the saline groups. In addition, TH-ir was reduced slightly in the MPTP + exercise group compared to MPTP alone.

nificantly reduced DAT-ir compared to the MPTP group (MPTP, 73.3 ± 4.5%; MPTP + exercise, 56.0 ± 6.3%; $P < 0.028$) (see Fig. 4 and compare Fig. 3I,J with Fig. 3K,L).

Exercise-Induced Changes in Tyrosine Hydroxylase Striatal Protein

Western immunoblotting with an antibody recognizing TH protein was carried out on striatal tissue at post-lesioning Day 4 and at the end of the exercise program (35 days after MPTP lesioning) (Fig. 5). Comparison of the Day 4 saline with the Day 4 MPTP lesioning showed a significant reduction in striatal TH protein due to MPTP lesioning (saline, 100 ± 3.0% compared to MPTP, 30.0 ± 4.5%). Comparison of the level of TH protein at the end of the running regimen (at Day 35) showed that there was no significant difference in TH immunoreactivity between any of the groups (saline, 100.0 ± 23.8%; saline + exercise, 105.8 ± 5.7%; MPTP, 83.3 ± 20.0%; MPTP + exercise, 56.0 ± 14.3%, $P + 3.72$). Although not significant (35 days after MPTP lesioning), MPTP groups (MPTP and MPTP + exercise) showed a slight reduction in TH immunoreactivity compared to the saline groups. In addition, TH immunoreactivity was reduced slightly in the MPTP + exercise group compared to that in the MPTP group.

Exercise-Induced Changes in Dopamine D1 and D2 Receptors

In situ hybridization histochemistry with probes recognizing either the D1 or D2 subtype of dopamine receptor was carried out on dorsal striatal tissue of mice from all groups. Compared to the saline group, expression of D1 mRNA was reduced as a result of exercise, MPTP lesioning, or both (saline, 100.0 ± 6.9%; saline + exercise, 51.9 ± 3.9%; MPTP, 50.1 ± 5.9%; MPTP + exercise, 48.6 ± 4.2%; $P < 0.0001$) (Fig. 6A). Comparison of the MPTP with the MPTP + exercise group showed no difference in the level of dopamine D1 mRNA expression. The expression of dopamine D2 mRNA was also reduced as a result of either exercise (saline + exercise) or MPTP lesioning (MPTP group) compared to that in the saline group (saline, 100.0 ± 7.6%; saline + exercise, 58.5 ± 6.4%; MPTP, 50.1 ± 5.7%; $P < 0.002$) (Fig. 6B). The combination of MPTP lesioning and exercise (MPTP + exercise group), however, resulted in no difference in dopamine D2 mRNA expression when compared to that in the saline group, but was increased significantly when compared to that in the MPTP group (MPTP + exercise, 95.9 ± 9.4%; $P < 0.005$).

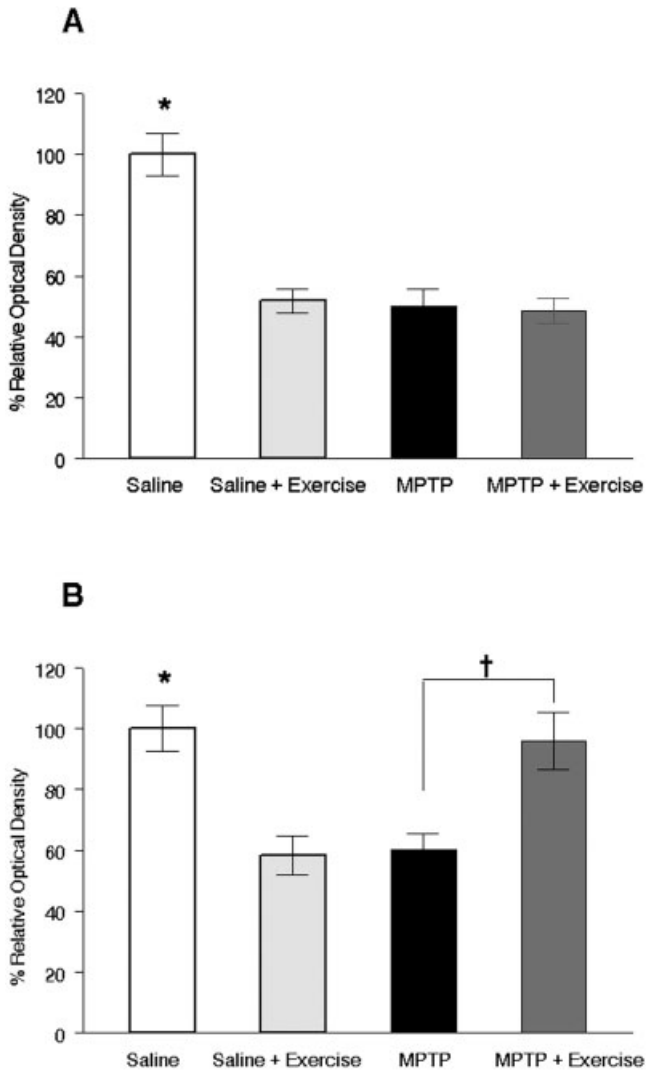

Fig. 6. Analysis of the relative striatal dopamine D1 and D2 receptor mRNA using in situ hybridization histochemistry. The relative optical density of autoradiographic grains above the dorsal striatum were determined from at least three mice from each treatment group using at least 12 sections/mouse. For comparison, the saline group was set arbitrarily at 100% and all other groups normalized against it for both dopamine receptors D1 and D2 mRNA. **A:** Compared to the saline group, expression of D1 mRNA was reduced significantly with exercise and MPTP lesioning (saline, 100.0 ± 6.9%; saline + exercise, 51.9 ± 3.9%; MPTP, 50.1 ± 5.7%; MPTP + exercise, 48.6 ± 4.2%; $P + 0.0001$). **B:** The expression of dopamine D2 mRNA was reduced as a result of either exercise (saline + exercise) or MPTP lesioning (MPTP group) compared to that in saline group (saline, 100.0 ± 7.6%; saline + exercise, 58.5 ± 6.4%; MPTP, 60.3 ± 5.3%; $P < 0.002$). The expression of dopamine D2 mRNA was increased significantly in the MPTP + exercise group when compared to that in the MPTP group (MPTP + exercise, 95.9 ± 9.4%; $P < 0.005$).

Exercise-Induced Changes in Nerve Terminal Glutamate Immunolabeling

Immunogold electron microscopy was used to determine the density of nerve terminal glutamate immunolabeling in mice from all groups at completion of the exercise program. Figure 7 shows representative images of asymmetrical (excitatory) synaptic contacts labeled for the neurotransmitter glutamate in mice from the saline group, saline + exercise group, MPTP group, and the MPTP + exercise group (Fig. 7A–D, respectively). There was a significant increase in the density of nerve terminal glutamate immunolabeling in the MPTP group compared to saline, (values are mean number of gold particles/μm^2 ± SEM: saline, 85.9 ± 3.6; MPTP, 135.3 ± 12.4; $P < 0.05$) (Fig. 8). Additionally, there was a significant decrease in the density of nerve terminal glutamate immunolabeling in the MPTP + exercise group compared to the MPTP group (MPTP, 135.3 ± 12.4; MPTP + exercise, 105.3 ± 4.5; $P < 0.05$). This decrease reached levels that were similar to the saline groups (mean number of gold particles/μm^2 ± SEM: saline, 85.9 ± 3.6; saline + exercise, 102.6 ± 3.7; MPTP, 135.3 ± 12.4; MPTP + exercise, 105.3 ± 4.5).

To determine the specificity of the change in the density of glutamate immunolabeling within the nerve terminal, the density of labeling within the presynaptic mitochondrial pool was quantified. There was no difference between any of the experimental groups (data not shown). Additionally, there were no changes in nerve terminal area between groups (data not shown).

Glutamate immunolabeling of the CA1 in the hippocampus was analyzed to confirm that the observed differences between groups in glutamate immunolabeling were specific to the striatum. This brain area was chosen because it also receives a significant glutamatergic input and is associated with spatial learning as opposed to the dorsolateral striatum that is associated primarily with motor function. There were no differences in the density of nerve terminal glutamate immunolabeling between any of the groups as shown in Figure 8B (values are mean number of gold particles/μm^2 ± SEM; saline, 100.0 ± 5.7; saline + exercise, 101.7 ± 7.9; MPTP, 88.4 ± 4.6; MPTP + exercise, 103.2 ± 6.6; $P < 0.49$).

DISCUSSION

The purpose of this study was to examine the effect of exercise on restoration of surviving dopaminergic neurons after completion of MPTP-induced cell death. The MPTP lesioning regimen used in our studies involves a series of four injections of 20 mg/kg (free-base) leading to a 60–70% loss of nigrostriatal dopaminergic neurons and a 90–95% depletion of striatal dopamine (Jackson-Lewis et al., 1995). Using this regimen, nigrostriatal cell loss is complete by Day 3 after MPTP administration and shows no further decline 30 days post-lesioning as determined by either unbiased stereologic counting techniques (Petzinger et al., in preparation) or section-sampling techniques (Jackson-Lewis et al., 1995). Despite the extent of cell loss, MPTP-lesioned mice display robust and reproducible return of striatal function 2–3 months after injury (Ricaurte et al., 1986; Jakowec et al., 2003). The levels of TH and DAT immunoreactivity (TH-ir and DAT-ir) are de-

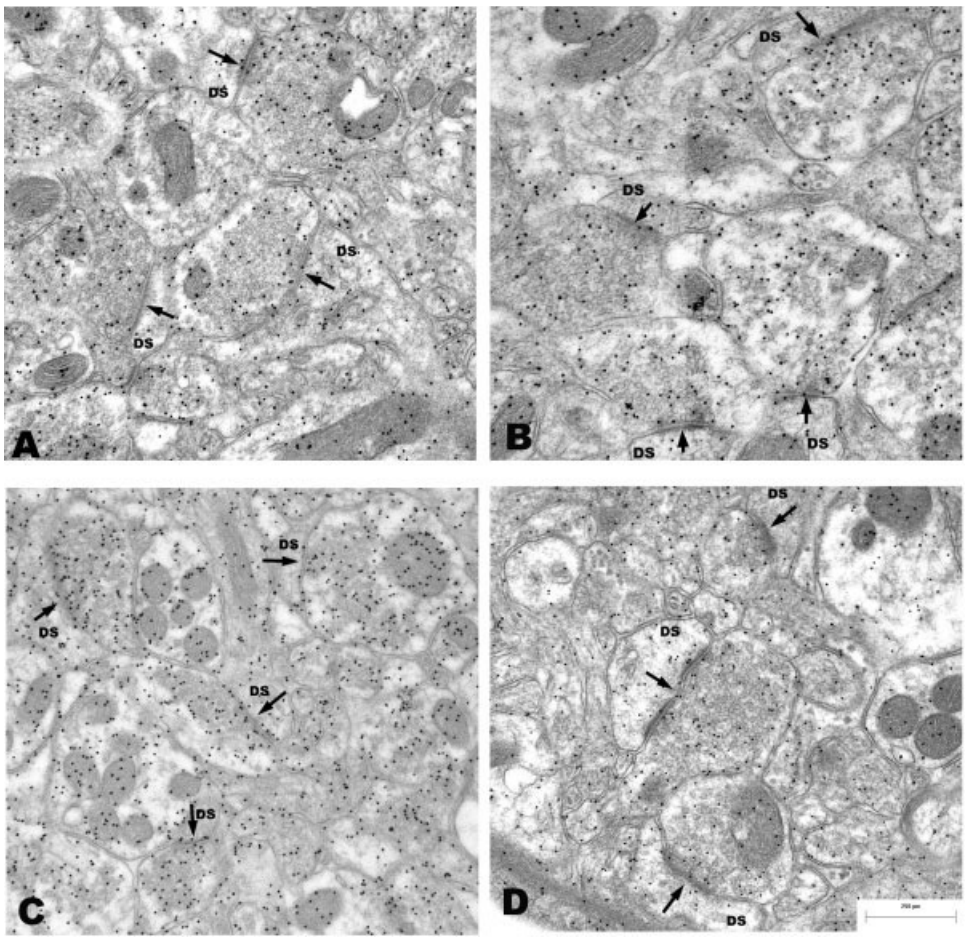

Fig. 7. Electron photomicrographs using the immunogold technique to localize an antibody against the neurotransmitter, glutamate, within the dorsolateral striatum. **A:** Saline group. Three nerve terminals are seen making an asymmetrical synaptic contact (arrows) with an underlying dendritic spine (DS). Within the nerve terminal are numerous 10-nm gold particles, indicating the location of the antibody. These gold particles are found overlying the round synaptic vesicles. **B:** Saline group that was exercised for 30 days, starting 4 days after the injection of saline. Note that the density of nerve terminal glutamate immunolabeling seems similar to that seen in the saline-treated group in A. **C:** MPTP-treated group was given an acute injection of the toxin (20 mg/kg × 4 injections every 2 hr) and then perfused with fixative 34 days later. Note the increase in the density of immunogold particles in all three nerve terminals compared to that observed in the saline group shown in A. **D:** MPTP-treated group that was exercised for 30 days, starting 4 days after the acute toxin treatment. Note that the density of glutamate immunogold labeling is similar to that seen in the saline-treated group in A. Scale bar + 0.25 μm.

creased to 30% of pre-MPTP-lesioned levels by Day 7 and return to 50–60% or more of pre-MPTP-lesioned levels within 30–60 days (Jakowec et al., 2003, 2004). These changes are part of molecular alterations underlying intrinsic neuroplasticity in this model (Jakowec et al., 2004) and are represented in this study by the MPTP nonexercised group. The saline group in this study served two purposes: (1) to show that the intensity of the exercise regimen was sufficient to induce a behavioral effect in non-lesioned controls; and (2) to compare the effect of exercise on the noninjured and injured brain.

Using a high-intensity (high velocity and duration) treadmill exercise paradigm in the MPTP-lesioned mouse, we have shown that exercise leads to behavioral recovery, specifically amelioration of initial deficits in running speed and duration compared to nonexercised MPTP-lesioned animals. Importantly, as would be expected by an effective training paradigm, the non-lesioned animals that exercised demonstrated enhanced performance compared to their non-lesioned, nonexercised counterparts. Specifically these behavioral differences between exercised and non-exercised mice consisted of the capability of the exercised saline mice to run at higher velocities after a 30-day treadmill-training program. We showed that MPTP-lesioned and non-lesioned mice could be forced to run at progressively faster speeds and longer durations and learn to associate a sensory stimulus with a behavioral response (i.e., maintaining a specific position on the treadmill). Over time, sensory feedback was no longer necessary for the animals to maintain a forward position, indicating that learning had occurred.

Although the MPTP-lesioned mouse displays subtle motor behavioral deficits that may not be evident under normal caging conditions, motor deficits become evident under specific task or environmental manipulations (Sedelis et al., 2001; Tillerson et al., 2002). Bradykinesia (slowness of movement) and fatigability (decreased endurance) were two behavioral deficits we observed initially in treadmill-exercised mice after MPTP lesioning. MPTP-lesioned mice were not capable of running at the same treadmill velocity as the saline + exercise mice in the first 18 days of running. By the end of the 30-day exercise program, the MPTP + exercise mice were running at a velocity near that of the saline + exercise group and greater than that of the saline (nonexercised) group (see Fig. 2C). The rate of change of velocity was greater in the

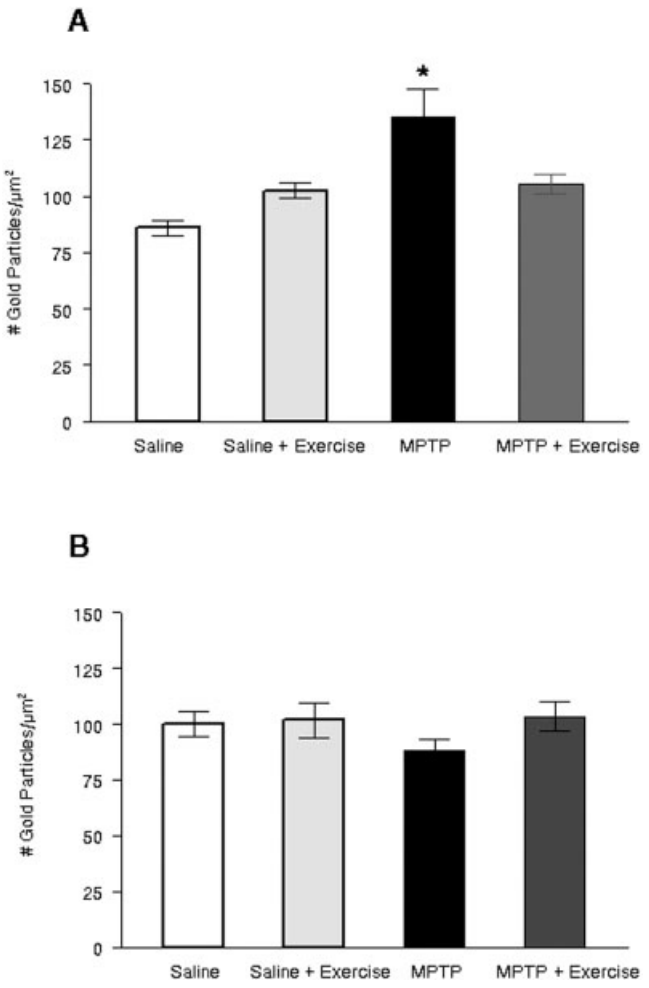

Fig. 8. Quantification of immunogold electron microscopy results. **A:** There was a significant increase in the density of synaptic glutamate immunolabeling within striatal nerve terminals making asymmetrical synaptic contacts after the acute administration of MPTP compared to the other treatment groups (mean number of gold particles/μm^2 $\pm$ SEM: saline, 85.9 $\pm$ 3.6; saline + exercise, 102.6 $\pm$ 3.7; MPTP, 135.3 $\pm$ 12.4; MPTP + exercise, 105.3 $\pm$ 4.5; $P < 0.05$). **B:** Using quantitative immunogold electron microscopy, there was no difference in the density of glutamate immunolabeling within nerve terminals making an asymmetrical synaptic contact within the CA1 region of the hippocampus between any of the treatment groups. $*P < 0.05$ compared to all the other groups as determined by the Fisher post-hoc test for comparison of multiple means.

injured animals compared to their nonlesioned exercised counterparts. This result is in accordance with exercise studies in cortically injured animals in which the effect of exercise is greater after injury. Injury may prime the system for adaptation perhaps through the induction of neurotrophic factors including brain-derived neurotrophic factor (BDNF) (Cotman and Berchtold, 2002; Gomez-Pinilla et al., 2002; Cohen et al., 2003).

Tillerson et al. (2003) also reported behavioral improvement after treadmill exercise in two rodent models of basal ganglia injury (the 6-OHDA rat and MPTP-lesioned mouse), which was associated with attenuation of dopamine loss (Tillerson et al., 2003). The investigators concluded that exercise might work largely through neuroprotective mechanisms because exercise was started within 12 hr of lesioning, and MPTP and 6-OHDA may take several days to complete cell death (Sauer and Oertel, 1994; Jackson-Lewis et al., 1995). Unlike the Tillerson et al. (2003) study, the focus of our study was to address the effect of high-intensity treadmill exercise on the neurorestoration of surviving neurons after MPTP lesioning. This was accomplished by: (1) initiating exercise 4 days after lesioning, a time period well after cell death is completed in this model; (2) continuing exercise over a 30-day period; and (3) progressively increasing treadmill velocity and duration over that period. Two additional differences between Tillerson et al. (2003) and the present study were the exercise parameters and the age of the animals. Our exercise parameters on young mice were of higher velocity, duration, and frequency and showed an effect on the saline + exercise group that was not seen in the Tillerson et al. (2003) study.

In addition to a behavioral effect, exercise resulted in decreased DAT-ir compared to the nonexercised groups. The effect of exercise on DAT-ir was even greater in the MPTP group. In the basal ganglia, the biosynthesis of dopamine is dependent on the enzyme tyrosine hydroxylase (TH) and the primary mechanism of clearance of dopamine from the extracellular space is through the dopamine transporter (DAT) (Gainetdinov et al., 2002; Mortensen and Amara, 2003). Several mechanisms have been shown to regulate DAT activity including: (1) gene and protein expression of transporter number; (2) phosphorylation activated through glutamate receptors such as the mGluR5 metabotropic receptor; and (3) internalization within endosomes mediated by dopamine (Perrone-Calano et al., 1996; Page et al., 2001). Alterations in DAT activity can influence the synaptic occupancy of dopamine. An intervention (such as exercise) that downregulates DAT-ir expression may therefore lead to behavioral improvement by increasing synaptic occupancy of dopamine. The downregulation of DAT-ir in our exercised animals could account for the superior running capabilities of both the MPTP and saline groups compared to the nonexercised groups. This interpretation is consistent with the findings of Meeusen et al. (1997) and others that report increased extracellular levels of dopamine with exercise. An alternative explanation for reduced DAT-ir in our exercised animals is the loss of nigrostriatal terminals where DAT normally resides. This does not seem likely because TH-ir, another marker of nigrostriatal terminal integrity, was not altered significantly and because exercised animals had superior running capability compared to their nonexercised counterparts, which would not be expected if cell death were ongoing. Studies are underway currently to investigate the possibility of cell death and terminal loss

including stereologic cell counting of substantia nigra pars compacta neurons and fiber density, respectively.

In saline animals, exercise suppressed dopamine D1 and D2 receptor mRNA levels. In the MPTP group, exercise seemed to have no effect on D1 but increased D2 mRNA levels. Of the dopamine receptor superfamily, D1 and D2 subtypes are the most prevalent in the striatum (REF). Activation of these receptors by dopamine leads to the release of neuropeptides from medium spiny neurons. Medium spiny neurons with D1 receptors express the neuropeptide preprotachykinin (PPT) and medium spiny neurons with D2 receptors express the neuropeptide preproenkephalin (PPE) (Gerfen, 2000). In the normal brain, D1 and D2 act synergistically and activation of both is required to elicit a motor response (Gerfen et al., 1995). In the lesioned basal ganglia, this synergy is lost and activation of either D1 or D2 may elicit a motor response. In addition, D2 activation alone in the injured state seems to elicit a more robust motor response that may be attributed to its heightened sensitivity after lesioning (LaHoste and Marshall, 1993). In our study, exercise seems to have a similar effect on both receptor subtypes in the saline animals that may reflect the synergy normally seen in the uninjured basal ganglia. This synergy, however, is lost in injury and affects each receptor subtype differently. The loss of synergy between the dopamine D1 and D2 receptors due to injury by MPTP is revealed in the context of exercise. One possible mechanism to explain the differential effect of exercise and injury on the dopamine receptors subtypes D1 and D2 may be through the action of glutamate. Glutamate has been shown to influence subtype-specific regulation of the dopamine receptors (see discussion below). The combination of the upregulation of D2 mRNA (leading to increased motor activity) along with the downregulation in DAT (leading to increased synaptic occupancy of dopamine) may explain the behavioral improvement seen in the MPTP + exercise mice. Studies are underway currently to localize the dopamine D2 receptor changes with exercise and to determine if D1 or D2 receptor antagonists or agonists affect the behavioral benefits of exercise.

Glutamate is the major excitatory neurotransmitter in the brain and plays an important role in motor behavior (Starr, 1995). Glutamate is stored within nerve terminals and upon release binds to a superfamily of receptors including the *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA), and kainic acid (KA) subtypes. In the striatum, the primary glutamatergic pathway is the corticostriatal input to the medium spiny neurons (Starr, 1995). One method for examining changes in glutamatergic neurotransmission is to measure alterations in glutamate storage within striatal nerve terminals using immunogold electron microscopy. We observed an increase in the density of nerve terminal glutamate immunolabeling in animals after MPTP lesioning. This increase was reversed by exercise to levels seen in the saline control groups. Additionally, this effect was specific to the lesioned dorsal-lateral striatum (an area associated primarily with motor function) because there was no alteration in CA1 glutamate terminals originating from either the Schaffer collaterals or from the contralateral hippocampus (an area associated primarily with learning and memory). In comparison to the MPTP-lesioned animals, no significant change in immunogold labeling was observed between the saline and saline + exercise groups. A change in glutamate terminal storage in the nonlesioned brain may require a higher intensity of exercise then used in the present studies (Meeusen et al., 1997).

Although glutamate levels were not measured in this study, Meshul et al. (2000) has shown an inverse relationship between terminal glutamate immunogold labeling and levels of glutamate within the synapse. An increase in the density of nerve terminal glutamate immunolabeling (as is seen with MPTP lesioning) may therefore reflect a decrease in the extracellular levels of striatal glutamate. Consequently, one hypothesis with respect to our results is that an effect of exercise in the MPTP-lesioned brain may be to increase the release of glutamate at the synapse, which that may alter dopamine receptor subtype expression or medium spiny neuron peptide expression (Cepeda et al., 1993; Cepeda and Levine, 1998; Liste et al., 1999). Using microdialysis in the 6-OHDA rat, Meeusen et al. (1997) showed an increase in extracellular glutamate with exercise (Meeusen et al., 1997; Bland et al., 1999).

Studies have shown that there are close interactions between glutamate and dopamine neurotransmission in mediating motor control (Starr and Starr, 1994; Starr, 1995; Starr et al., 1997). The striatal medium spiny neuron is thought to be the site for integrating these interactions. Exercise may either directly affect the medium spiny neuron or indirectly influence its afferents. For example, expression of the immediate early gene cFos (a marker of cell activation) and the neuropeptides preprotachykinin and preproenkephalin in medium spiny neurons are altered by exercise (Cepeda et al., 1993; Liste et al., 1999). Altered expression of these markers in medium spiny neuron activity can be blocked by either glutamate or dopamine receptor antagonists or through denervation. The present study has shown changes in both glutamate and dopamine systems. We do not yet know, however, if glutamate and dopamine changes are dependent or mutually exclusive of each other. To test the degree of dopamine–glutamate interactions with exercise, we are conducting additional studies with exercise in MPTP and saline mice administered either glutamate or dopamine antagonists. If the suppression of DAT-ir seen in our studies can be blocked by administration of a glutamate antagonist during exercise, it would support the hypothesis that glutamatergic neurotransmission is important in regulating exercise-induced changes in dopamine function. Furthermore, dopamine receptor-specific agonists and antagonists targeting either D1 or D2 will test whether the alterations in glutamate immunolabeling seen in our studies are dependent on dopamine neurotransmission.

In conclusion, exercise may be both neuroprotective and neurorestorative in the injured basal ganglia. It has been shown previously that initiating exercise at or during the time of neurotoxin-induced cell death is neuroprotective by attenuating striatal dopamine loss (Cohen et al., 2003; Tillerson et al., 2003). Our studies show that a high-intensity treadmill exercise paradigm initiated after the period of neurotoxin-induced cell death is neurorestorative as demonstrated through its beneficial effect on motor behavior. Alterations in both dopaminergic and glutamatergic neurotransmission in response to exercise may underlie the molecular mechanisms of this effect. The potential impact of this study is that exercise may not only play a role in prevention of Parkinson's disease but in restoring function in individuals who have been diagnosed with Parkinson's disease.

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