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## Exercise Elevates Dopamine D2 Receptor in a Mouse Model of Parkinson's Disease: In Vivo Imaging with [<sup>18</sup>F]Fallypride

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### Abstract

The purpose of the current study was to examine changes in dopamine D2 receptor (DA-D2R) expression within the basal ganglia of MPTP mice subjected to intensive treadmill exercise. Using Western immunoblotting analysis of synaptoneurosomes and *in vivo* positron emission tomography (PET) imaging employing the DA-D2R specific ligand [<sup>18</sup>F]fallypride, we found that high intensity treadmill exercise led to an increase in striatal DA-D2R expression that was most pronounced in MPTP compared to saline treated mice. Exercise-induced changes in the DA-D2R in the dopamine-depleted basal ganglia are consistent with the potential role of this receptor in modulating medium spiny neurons (MSNs) function and behavioral recovery. Importantly, findings from this study support the rationale for using PET imaging with [<sup>18</sup>F]fallypride to examine DA-D2R changes in individuals with Parkinson's Disease (PD) undergoing high-intensity treadmill training.

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## Keywords

positron emission tomography; basal ganglia; neuroplasticity; treadmill exercise

Exercise improves motor performance in patients with Parkinson's disease (PD).<sup>1-3</sup> Animal models, such as the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse, provide a critical tool to investigate the molecular mechanisms of exercise-induced improvement in motor behavior.<sup>4-6</sup> The dopamine D1 and D2 receptors (DA-D1R and DA-D2R) are the primary targets of dopamine on striatal medium spiny neurons (MSNs) and modulate physiological properties and cellular signaling. Specifically, the DA-D2R plays a major role in long-term depression (LTD), a form of synaptic plasticity that involves integration of glutamatergic and dopaminergic neurotransmission leading to the encoding of motor function in the dorsolateral striatum. Given the role of the DA-D2R in motor control, we sought to examine whether exercise enhanced improvement in motor function is due in part to an increase in striatal DA-D2R expression.

Positron emission tomography (PET)-imaging with DA-D2R radiotracers offers the ability to carry out longitudinal studies on the effect of exercise in humans. Previous studies with aerobic exercise have attempted to measure dopamine release in normal individuals<sup>7</sup> and no change in the binding of [<sup>11</sup>C]raclopride was observed, leading the authors to suggest that little change in dopamine levels occurred. However, the effects of exercise on DA-D2R expression and synaptic activity has not been studied. The PET-imaging ligand [<sup>18</sup>F]fallypride is an excellent tool to examine this due to its high affinity and specificity for both DA-D2R and DA-D3R, and unlike [<sup>11</sup>C]raclopride, it is not readily displaced by baseline levels of endogenous dopamine.<sup>7-10</sup> This was confirmed by reserpine pretreatment of animals (to deplete endogenous dopamine) that had no effect on [<sup>18</sup>F]fallypride binding,<sup>9,11</sup> but significantly increased [<sup>11</sup>C]raclopride binding<sup>8</sup> that was attributed to a change in the apparent binding affinity ( $K_d$ ) rather than receptor number ( $B_{max}$ ).

As the binding potential (BP) of [<sup>18</sup>F]fallypride is resistant to changes due to depletion of dopamine, suggesting little effect on its  $K_d$  or  $B_{max}$  at baseline or depleted state, we used [<sup>18</sup>F]fallypride to test our hypothesis that DA-D2R expression increases in the MPTP mouse model with intensive exercise.<sup>9,10,12,13</sup> Furthermore, to support our PET imaging measures, we used the complementary technique of Western immunoblot analysis of synaptoneurosomal preparations to measure changes in DA-D2R protein expression at the level of synapse in the same animals. We report here the effects of exercise on DA-D2R expression and [<sup>18</sup>F]fallypride in groups of mice treated with either saline or MPTP.

## METHODS

### Animals, Treatment Groups, and MPTP Administration

Male C57BL/6 mice 8 weeks old (Charles River Laboratories, Wilmington, MA) were group-housed in a temperature-controlled room under 12 h light/12 h dark cycle. All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals as approved by the USC IACUC. A total of 164 mice were used in four treatment groups: (1) saline (n = 42), (2) saline plus exercise (n = 55), (3) MPTP (n = 57), and (4) MPTP plus exercise (n = 42). For lesioning, mice received four intraperitoneal injections of 20 mg/kg MPTP (free-base; Sigma-Aldrich, St. Louis, MO) dissolved in 0.9% saline, at 2-h intervals or four intraperitoneal injections of 0.1 ml 0.9% NaCl as control. Lesioning was validated by HPLC analysis of striatal dopamine levels. At 10 days post-MPTP administration, there was 82.2% dopamine depletion in MPTP mice ( $48.0 \pm 8.4$  ng/mg of protein) compared with saline mice ( $269.5 \pm 24.9$  ng/mg of protein). At the end of the

study, there was no significant difference in striatal dopamine levels between MPTP plus exercise mice ( $69.8 \pm 11.7$  ng/mg of protein) compared with MPTP ( $77.9 \pm 12.0$  ng/mg of protein). However, there was a significant increase of striatal dopamine in saline plus exercise mice ( $315.2 \pm 9.0$  ng/mg of protein) compared with saline ( $246.9 \pm 19.8$  ng/mg of protein) ( $F_{(3,16)} = 7.78$ ;  $P < 0.05$ ).

### Treadmill Exercise

Exercise started 5 days after lesioning. Mice from the two exercise groups (saline plus exercise and MPTP plus exercise) were trained to run on a 100-cm motorized treadmill (Exer 6M, Columbus Instruments, OH) at incremental speeds for 6 weeks (5 days/week) to reach duration of 60 min/day and speed of 18–20 m/min.<sup>5,6</sup>

### Magnetic Resonance Imaging

A three-dimensional volumetric T1-weighted magnetic resonance (MR) image of the mouse brain was obtained with a 7-T micro-MRI system (Bruker Biospin, Billerica, MA). Parameters of image acquisition were: TE = 46.1 ms, TR = 6292.5 ms, 0.4-mm slice thickness, 0.45-mm interslice thickness,  $128 \times 128 \times 128$  matrix size.

### Radiochemistry

Synthesis of [<sup>18</sup>F]fallypride was performed as previously described through nucleophilic substitution reaction of the tosyl precursor with [<sup>18</sup>F] using a custom-made radiochemistry apparatus.<sup>12</sup> Purification was achieved by reverse-phase HPLC on a C8(2) Phenomenex Luna column using acetonitrile and sodium phosphate buffer as mobile phase (55:45). UV absorbance was measured at 254 nm and AUFS 0.05. Radioactive peak (retention time 17 min) corresponding to [<sup>18</sup>F]fallypride, was collected and solvent removed on a rotary evaporator. The final product was tested for pyrogenicity, sterility, pH, and removal of organic solvents by gas chromatography. Specific activity and radiochemical purity was assessed with a Waters HPLC system using a C8(2) Phenomenex Luna analytical. Specific activity was in the range of 3,000–12,000 Ci/mmol.

### PET Measurements and Image Analysis

Twenty mice were used for PET imaging ( $n = 6$  saline;  $n = 3$  saline plus exercise;  $n = 5$  MPTP; and  $n = 6$  MPTP plus exercise). Scans were acquired with a Concorde microPET R4 scanner (CTI Concorde Microsystems, Knoxville, TN) with a 60-min list mode acquisition protocol after 20-min transmission scan for attenuation correction with a <sup>68</sup>Ge source. [<sup>18</sup>F]fallypride (10.92–11.28 MBq) was injected via the tail vein (single bolus) at the start of the emission scan. Mice were anesthetized with 2% isoflurane and 98% oxygen. The dynamic list mode data were sorted to sinograms with 26 frames ( $6 \times 20$  sec,  $4 \times 40$  sec,  $6 \times 1$  min, and  $10 \times 5$  min) and reconstructed by two iterations of OSEM (ordered subsets expectation maximization) followed by 18 iterations of the MAP (maximum a posteriori) reconstruction algorithm.<sup>14</sup> Reconstructed images were cropped to contain the head and linearly interpolated in the Z-direction to produce a  $128 \times 128 \times 63$  image with isotropic  $0.4 \times 0.4 \times 0.4$  mm<sup>3</sup> voxels. High-resolution binding potential (BP) images of the striatum were computed from the reconstructed dynamic images using a multilinear tissue reference model<sup>15</sup> and Logan plots<sup>16</sup> with high activity in the striatum and very low activity in the cerebellum (reference region). Anatomical regions of interest (striatum and cerebellum) were manually defined in both hemispheres in PET images coregistered with MRI using Rview (version 8.21Beta).<sup>17</sup> Quantification of specific binding of [<sup>18</sup>F]fallypride in the mouse striatum was performed using the BP value that provides a measure of the ratio of specific/nonspecific binding at equilibrium.<sup>18,19</sup> To demonstrate binding specificity in the striatum, four mice were harvested 60 min after ligand injection, brains quickly frozen in

liquid nitrogen, sectioned at 30- $\mu$ m thickness, and sections apposed to a phospho-imager (Typhoon 9200, GE Healthcare Inc., Piscataway, NJ) (Fig. 1). Studies have shown that [ $^{18}$ F]fallypride binds specifically to the DA-D2R, and as very little DA-D3R is in the striatum, binding indicates DA-D2R occupancy.<sup>9,10,12,13</sup>

### Tissue Collection for HPLC and Protein Analysis

At the end of the study, brains were quickly removed and dorsal striatum dissected fresh corresponding to anatomical regions from bregma 1.2 to 0.6 with the corpus callosum as dorsal border, the lateral aspect of the corpus callosum as lateral border, and above the anterior commissure as the ventral border.<sup>20</sup>

### HPLC Analysis of Dopamine and Its Metabolites

Dopamine levels in striatal homogenates (n = 4 per group) were determined by HPLC with electrochemical detection.<sup>6</sup> The system consisted of an ESA auto-sampler (ESA, Chelmsford, MA) equipped with a 150  $\times$  3.2 mm reverse-phase C-18 column (3 $\mu$ m diameter) and a CoulArray 5600A (ESA, Chelmsford, MA), equipped with a four-channel analytical cell with potentials set at -75, 50, 220, and 350 mV.

### Western Immunoblot Analysis

Exercise effect on synaptic expression of DA-D1R and DA-D2R was analyzed in synaptoneurosome preparations made fresh from eight pooled dorsolateral striatum.<sup>21</sup> This procedure was performed on three sets of mice for a total of 24 mice per experimental group (n = 3 preps per group). Relative expression of proteins for DA-D1R (~50 kDa), DA-D2R (~50 kDa), tyrosine hydroxylase (58 kDa), dopamine transporter (68 kDa), and  $\alpha$ -tubulin (50 kDa) (as loading control) were analyzed by Western immunoblot<sup>22</sup> using commercially available primary antibodies (rabbit polyclonal and mouse monoclonal antibodies, Millipore, Temecula, CA). Protein bands were visualized by affinity purified goat anti-rabbit or anti-mouse secondary antibodies conjugated to IRDye<sub>680</sub> or IRDye<sub>800</sub> (Rockland, Gilbertsville, PA). Fluorescent signal was detected by scanning the filter in a LI-COR Odyssey near infrared imaging platform and quantified using Odyssey 2.1 software (LI-COR Biotechnology, Lincoln, NE). The results are shown as relative expression levels compared with the saline group (set to 100%).

### Statistical Analysis

Differences between groups in BP of [ $^{18}$ F]fallypride, DA-D1R, and DA-D2R protein levels were analyzed using two-way analysis of variance (ANOVA) with treatment as between subject factor (saline vs. MPTP), and exercise as within subject factor (no exercise vs. exercise). For maximal treadmill speed test, time was used as between subject factor (week 1, 2, etc.) and treatment was used as within subject factor (saline vs. MPTP). The Bonferroni post hoc test was used to correct for multiple comparisons when assessing significance of interest. Significance level was set to  $P < 0.05$ . To explore the practical significance of group differences, an estimate of the magnitude of the differences between groups was calculated using the effect size (ES) ( $ES = \text{Mean}_{\text{Group 1}} - \text{Mean}_{\text{Group 2}} / SD_{\text{pooled}}$ ). The ES reflects the impact of treatment within a population of interest and is reported according to established criteria as small (<0.41), medium (0.41–0.70), or large (>0.70).<sup>23</sup> Analysis was performed using Prism5 for Windows (GraphPad, San Diego, CA).

## RESULTS

### High-Intensity Treadmill Exercise Improved Motor Behavior in MPTP-Lesioned Mice

Prior to MPTP-lesioning and start of exercise, average baseline velocities of all mice in two exercise groups were similar (saline plus exercise:  $11.7 \pm 1.1$  m/min, and MPTP plus exercise:  $11.2 \pm 1.1$  m/min). Daily exercise for 6 weeks improved maximal treadmill velocities in both exercise groups with the saline plus exercise mice displaying significantly greater maximal velocity compared to the MPTP plus exercise mice in weeks 1 through 4 (Fig. 2). However, MPTP plus exercise mice had similar maximal treadmill speeds as saline plus exercise mice in week 5 (MPTP plus exercise:  $17.2 \pm 3.6$  m/min and saline plus exercise:  $22.0 \pm 1.5$  m/min) and week 6 ( $19.2 \pm 1.2$  m/min and  $22.2 \pm 0.9$  m/min, respectively). As previously reported, MPTP-lesioned mice that did not undergo treadmill training displayed no spontaneous recovery of motor behavior with their maximal velocity of  $7.0 \pm 0.3$  m/min at the end of the 6-week exercise period.<sup>5</sup>

### High-Intensity Treadmill Exercise Increased Striatal DA-D2R but not DA-D1R Protein

High-intensity treadmill exercise differentially affected DA-D2R and DA-D1R levels in synaptoneurosomal preparations from the dorsal striatum as shown by Western blot analysis (Fig. 3). MPTP plus exercise mice had 48.8% increase in striatal DA-D2R compared with MPTP mice (Fig. 3B), and significant interaction between exercise and MPTP lesioning on DA-D2R protein level ( $F_{(1,8)} = 6.0$ ;  $P < 0.05$ ). Conversely, there was no exercise effect on DA-D1R protein levels between the groups (Fig. 3A;  $F_{(1,8)} = 0.1$ ,  $P = 0.78$ ). MPTP lesioning alone did not significantly alter either DA-D2R ( $F_{(1,8)} = 0.0$ ;  $P = 0.88$ ) or DA-D1R expression ( $F_{(1,8)} = 0.0$ ;  $P = 0.92$ ). In addition, two different protein markers of midbrain dopaminergic fibers integrity, tyrosine hydroxylase (TH; Fig. 3C) and dopamine transporter (DAT; Fig. 3D), showed that MPTP significantly decreased striatal TH protein ( $F_{(1,8)} = 757.3$ ;  $P < 0.05$ ) and DAT expression ( $F_{(1,8)} = 218.0$ ;  $P < 0.05$ ).

### High-Intensity Treadmill Exercise Increased Striatal [<sup>18</sup>F]Fallypride Binding Potential (BP)

While Western immunoblotting analysis of receptor protein expression measured total antibody epitopes (both surface and internal cellular stores), *in vivo* PET-imaging with the high-affinity DA-D2R-specific radioligand [<sup>18</sup>F]fallypride can delineate the effects of exercise on availability of DA-D2R to bind ligand (Fig. 4). Statistical analysis revealed that there was a significant effect of exercise ( $F_{(1,16)} = 12.3$ ;  $P < 0.05$ ) as well as MPTP lesion ( $F_{(1,16)} = 160.3$ ;  $P < 0.05$ ) with no significant interaction between MPTP and exercise ( $F_{(1,16)} = 3.5$ ;  $P = 0.07$ ) on [<sup>18</sup>F]fallypride BP. The Bonferroni post hoc analysis showed significant difference in BP values between MPTP and MPTP plus exercise mice ( $t = 1.1$ , Df = 1, 16;  $P < 0.01$ ), and no significant difference between saline and saline plus exercise mice ( $t = 4.1$ , Df = 1, 16;  $P > 0.05$ ). Specifically, MPTP plus exercise mice had a 73.1% increase in [<sup>18</sup>F]fallypride BP compared to MPTP mice (average BP values for MPTP plus exercise:  $7.1 \pm 0.7$ ; average BP values for MPTP mice:  $4.1 \pm 0.3$ ) (Fig. 4B). In addition, saline plus exercise mice had an 8.2% increase in [<sup>18</sup>F]fallypride BP ( $13.2 \pm 1.0$ ) compared to saline mice ( $12.2 \pm 0.3$ ). Consistent with these findings “effect size” calculations revealed a larger exercise effect between MPTP groups (ES = 2.61) than that observed between the saline groups (ES = 0.94).

## DISCUSSION

This study demonstrates that high-intensity treadmill exercise leads to an increase in [<sup>18</sup>F]fallypride BP (DA-D2R availability) in the striatum of MPTP treated mice. Conversely, there was no significant change in total striatal dopamine levels between MPTP plus exercise compared with MPTP no exercise mice. [<sup>18</sup>F]fallypride is a highly selective DA-



D2/D3R antagonist whose BP reflects an *in vivo* measure of available receptors ( $B_{max}$ )/binding affinity ( $K_d$ ). As DA-D2Rs are the predominant dopamine receptor subtype within dorsal striatum, an exercise-induced increase in [ $^{18}\text{F}$ ]fallypride BP represents an increase in DA-D2R number and is supported by an increase in protein expression using Western immunoblotting and our previous studies showing an increase in striatal DA-D2R mRNA transcript expression using *in situ* hybridization histochemistry.<sup>5</sup> This interpretation of BP elevation is further supported by the fact that displacement of [ $^{18}\text{F}$ ]fallypride by dopamine is not likely to occur in MPTP mice as dopamine levels remain low.<sup>24</sup> Hence, changes in apparent binding affinity ( $K_d$ ) are negligible and are unlikely to effect BP. The enhanced effect of exercise in MPTP mice may reflect an attempt of the injured brain to optimize dopaminergic neurotransmission through increased receptor number while dopamine levels remain depleted. Increased responsiveness of MPTP mice to exercise reveals a greater potential of the injured versus the intact brain to undergo neuroplasticity, which may not be essential when striatal circuitry is intact. The fact that dopamine levels do not change significantly with exercise in MPTP mice suggests that compensatory changes in DA-D2R are critical for exercise related improved motor performance.

Using PET imaging, we observed a decrease in DA-D2R BP after MPTP lesioning relative to saline-treated mice. This was in contrast to Western immunoblotting in which no change in DA-D2R protein expression was observed. The DA-D2R exists in a dynamic equilibrium between surface and intracellular compartments, with the latter not generally available to binding to PET radioligands. In the dopamine-depleted state, compensatory mechanisms may lead to changes in the intracellular pool for DA-D2R, which may be unavailable for [ $^{18}\text{F}$ ]fallypride binding but yet available for detection in Western immunoblotting.

Unlike our findings, a compensatory increase in the DA-D2R has been reported in individuals with PD and after administration of MPTP in nonhuman primates, or 6-OHDA in rats.<sup>25</sup> In the literature, the loss of DA-D2Rs is reportedly due to the degeneration of dopaminergic neurons, whereas the increase in DA-D2Rs results from increased expression on remaining dopaminergic terminals and/or increased synthesis within striatopallidal neurons or cholinergic interneurons. This discrepancy between our PET study, and those of the literature, may be due to differences in the severity of the lesion between studies.<sup>11</sup> Specifically, the loss of a greater number of presynaptic DA-D2Rs through MPTP-induced cell loss may be sufficient to offset any postsynaptic compensatory changes induced by the lesion alone. Alternatively, our inability to observe an increase in DA-D2R BP and expression levels in MPTP (non-exercise) mice may be due to a modest recovery of dopamine levels at the end of the study (82% dopamine depletion at 10 days versus 68% depletion at 42 days postlesion). However, this is unlikely as the MPTP plus exercise mice, which also displayed a small recovery of dopamine (not significantly different from the MPTP no exercise mice) had increase of DA-D2R BP.

The majority of DA-D1Rs and D2Rs are expressed on dendritic spines of MSNs with additional receptors expressed on cholinergic interneurons and terminals of glutamatergic and dopaminergic neurons originating from the cortex (or thalamus) and substantia nigra pars compacta, respectively.<sup>26</sup> A major role of dopamine is to modulate corticostriatal or thalamostriatal glutamatergic neurotransmission at the MSN. Glutamatergic neurotransmission is enhanced through DA-D1Rs and diminished through DA-D2Rs.<sup>27–29</sup> Under conditions of dopamine depletion, spines and synaptic connections are selectively lost on DA-D2R containing MSNs of the indirect pathway.<sup>30</sup> This loss is accompanied with a hyperexcitability state within the MSNs due to increased glutamatergic corticostriatal neurotransmission.<sup>31–33</sup> In animal models of PD, this increased glutamatergic drive correlates with parkinsonian-like motor behavior.<sup>34</sup> Attenuation of this hyperexcitable state through the application of dopamine or its agonists leads to reversal of parkinsonian motor

deficits.<sup>35,36</sup> In light of these reports and our findings, we hypothesize that the benefits of high-intensity exercise are to enhance dopaminergic signaling through increased DA-D2R expression in the indirect pathway (but not the DA-D1R direct pathway) and to improve motor function through suppression of glutamatergic excitability.

The primary conclusion of our study is that exercise in the form of intensive treadmill running facilitates neuroplasticity through increased expression of striatal DA-D2Rs, a process most evident in the injured brain. Based on our findings, a noninvasive PET-imaging approach with [<sup>18</sup>F]fallypride can be used to investigate whether intensive treadmill exercise also leads to changes in the DA-D2R in individuals with PD. Our study highlights the value of preclinical research in animal models of dopamine depletion and the importance of translational research for providing both rationale and insight toward understanding imaging and exercise studies in individuals with PD.

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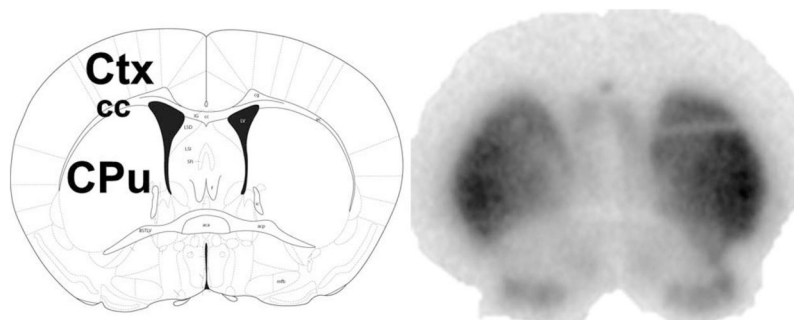
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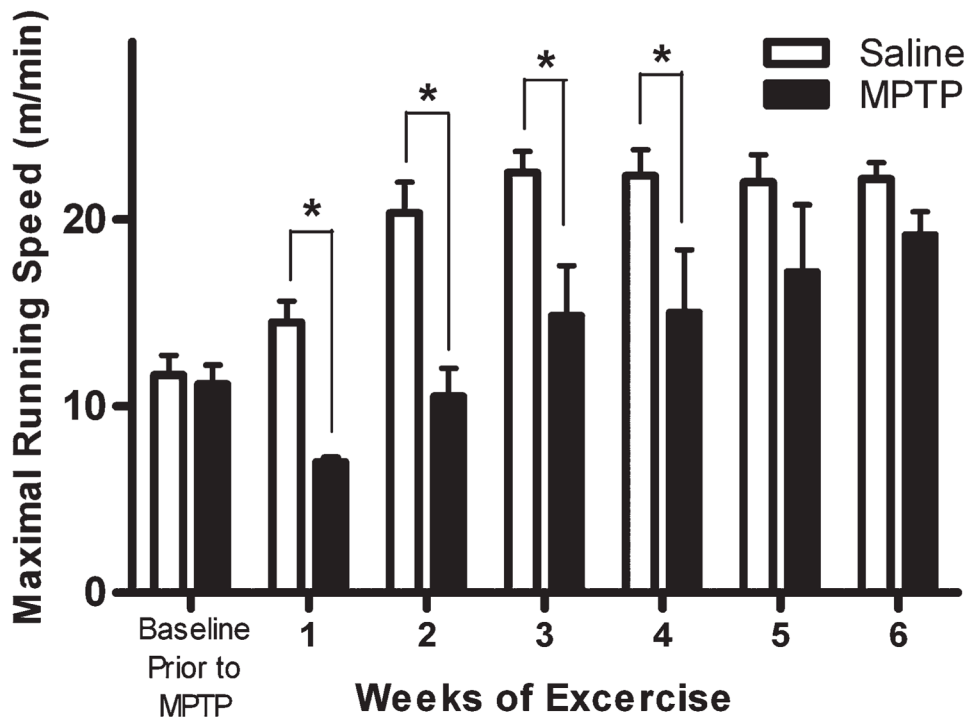
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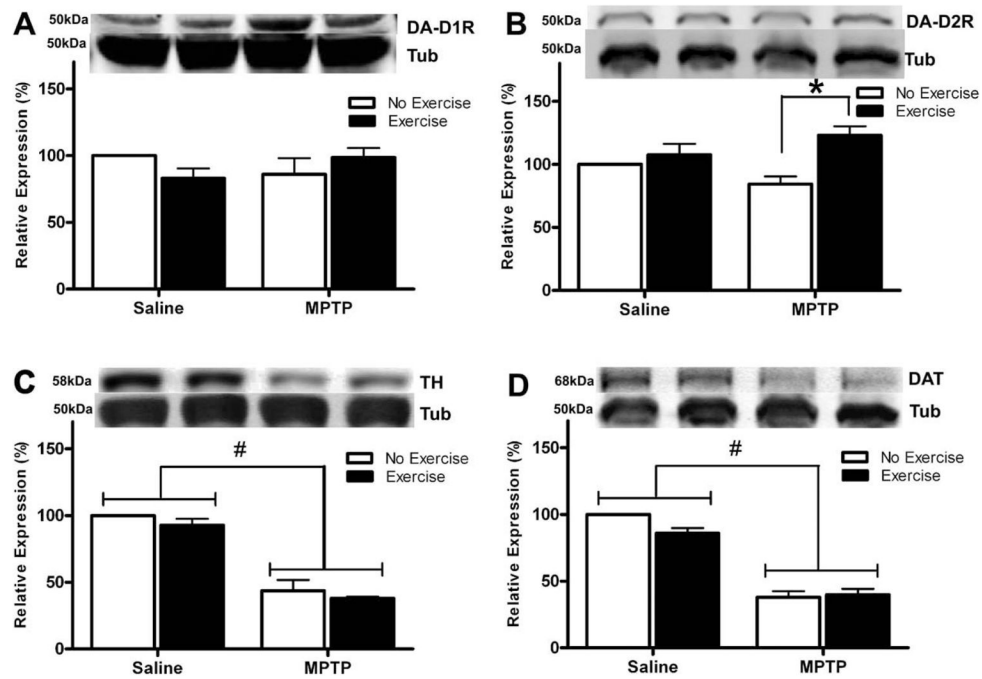
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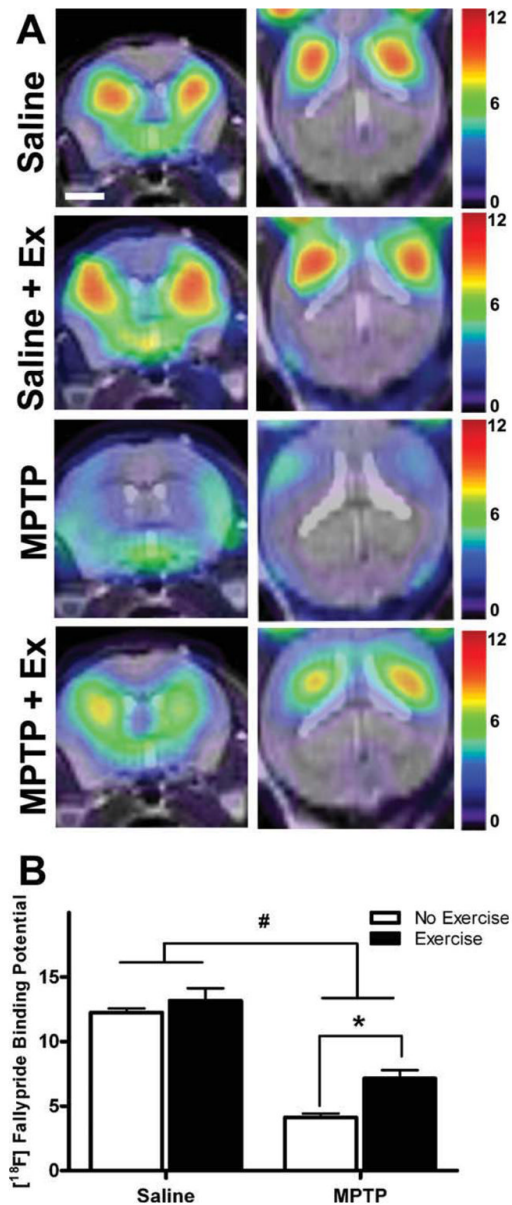
**FIG. 1.** [ $^{18}\text{F}$ ]Fallypride shows high binding specificity to the mouse striatum. The left panel shows an anatomical rendering of the coronal section at approximate level bregma 0.20. The right panel shows a representative autoradiograph with intensive labeling corresponding to the striatum.



**FIG. 2.** Exercise improves motor behavior in the MPTP mouse. The maximum running speed of saline ( $n = 12$ ) and MPTP ( $n = 12$ ) mice on the motorized treadmill was tested at the end of each week. The baseline treadmill velocities were measured prior to MPTP lesioning. By the end of the running period, there was no difference in velocity between saline and MPTP mice. Results are shown as mean  $\pm$  SEM. Data were analyzed by two-way ANOVA with repeated measures; the symbol “\*” represents significance level  $P < 0.05$ . Significant differences in maximal treadmill velocity were seen at weeks 1 through 4.

**FIG. 3.**

Exercise selectively up-regulates DA-D2R but not DA-D1R striatal protein. Panel (A) shows Western immunoblot analysis of synaptoneurosomes from the dorsal striatum for DA-D1R protein. There was no statistically significant difference between treatment groups. Panel (B) shows Western immunoblot analysis of synaptoneurosomes from the dorsal striatum for DA-D2R protein. MPTP plus exercise mice showed elevated DA-D2R protein compared to MPTP mice. (C) Analysis of tyrosine-hydroxylase (TH) protein and (D) dopamine transporter (DAT) protein (markers of midbrain dopaminergic terminals) in synaptoneurosomes from four different groups. MPTP treatment significantly decreased levels of TH and DAT proteins in dorsal striatum. These data were generated in three separate experiments, each consisting from pooled tissue ( $n = 8$  brains/group). Results are shown as mean  $\pm$  SEM. The symbol “#” represents significant MPTP effect ( $P < 0.05$ ), and the symbol “\*” represents significant effect of exercise in MPTP groups as shown by two-way ANOVA with Bonferroni post hoc correction ( $P < 0.05$ ).



**FIG. 4.** Exercise selectively increases [<sup>18</sup>F]fallypride binding potential (BP) in the striatum of MPTP mice. Panel (A) shows [<sup>18</sup>F]fallypride BP representative images in the coronal orientation (left side) and horizontal orientation (right side). The scale bar to the right shows relative BP intensity with high in red and low in blue. Panel (B) graphically depicts the BP data showing that MPTP reduced [<sup>18</sup>F]fallypride BP, while exercise increased [<sup>18</sup>F]fallypride BP in both saline and MPTP mice. The numbers of mice used for each group were saline (n = 6), saline plus exercise (n = 3), MPTP (n = 5), and MPTP plus exercise mice (n = 6). Results are shown as mean ± SEM. The symbol “#” represents significance level of MPTP effect (*P* < 0.05), and “\*” represents the effect of exercise in MPTP groups as shown by two-way ANOVA with Bonferroni post hoc correction (*P* < 0.01).