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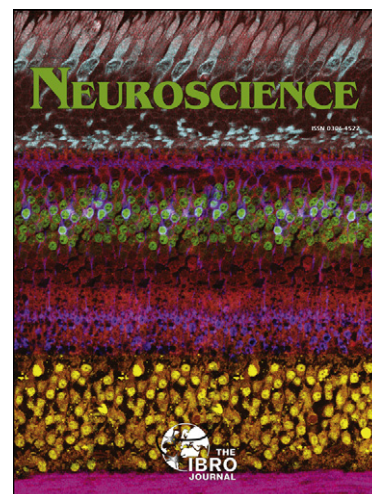
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ABSTRACT

L-3,4-dihydroxyphenylalanine (L-DOPA) alleviates the motor symptoms of Parkinson's disease (PD), but its long-term use is associated with underirritable dyskinesia. We now tested whether exercise can attenuate these L-DOPA-induced dyskinesia (LID). We tested the effects of exercise on L-DOPA-induced dyskinesia (LID) in 6-OHDA-hemiparkinsonian mice. Animals were treated with L-DOPA/benserazide (25/12.5 mg/kg, i.p.) without and with possibility to exercise (running wheel) during two weeks. Exercise drastically prevented the development of LID, and its associated aberrant striatal signaling, namely the hyperphosphorylation of dopamine and cAMP regulated phosphoprotein 32 kDa (DARPP-32) protein and c-Fos expression. Our results indicate that exercise can partially prevent the development of LID through the normalization of striatopallidal dopaminergic signaling.

Key-words: DARPP-32; dyskinesia; exercise; L-DOPA; Parkinson's disease; 6-OHDA.

1. INTRODUCTION

The nigrostriatal pathway provides the source of dopamine (DA) to the striatum, as part of basal ganglia motor loop, which is involved in the control of movements (Holschneider et al., 2007). In Parkinson's disease (PD), nigrostriatal neurons degenerate and supplementation with oral L-3,4-dihydroxyphenylalanine (L-DOPA), a DA precursor, is the main clinical strategy to counteract the motor symptoms of PD and improve rehabilitation (Blonsky and Minnigh, 1970, Mikkelsen et al., 1972). However, long-term L-DOPA treatment induces the development of severe dyskinesia (Parkes, 1981), an important socio-economic cause of distress for PD patients (Maurel et al., 2001). The pathophysiology of L-DOPA-induced dyskinesia (LID) remains unknown, but neurochemical studies have pinpointed the association of LID with a sustained activation of cdk5 (cyclin-dependent kinase 5) and DARPP-32 signaling (DA- and cAMP-regulated neuronal phosphoprotein, 32 kDa), and an increased expression of FosB and prodynorphin in striatopallidal GABAergic medium-sized spiny neurons (MSNs) (Andersson et al., 1999, Picconi et al., 2003). Albeit LID is common, they are difficult to treat, since therapeutic options to manage dyskinesia are scarce. Amantadine, a glutamate NMDA receptor antagonist, is the only approved pharmacotherapy (Lundblad et al., 2005),

as an alternative to the expensive and complex deep brain stimulation or continuous infusion of dopaminergic drugs (Cenci, 2007).

Along with L-DOPA, exercise can also stimulate and normalize motor function in animal models of PD (Aguiar et al., 2009, VanLeeuwen et al., 2010). In particular, exercise reduces stereotypic movements (Klusha et al., 1983, Dey and Singh, 1992, Teixeira et al., 2008), the phosphorylation of DARPP-32 (Aguiar et al., 2010) and the expression of c-Fos in the murine striatum (Liste et al., 1997). However, since the effects of exercise on LID are unknown, we now investigated the effects of exercise in the development of LID in an experimental model of PD.

2. METHODS

This study was approved by the UFSC Ethics Committee, which follows National Research Council principles (USA). All drugs were purchased from Sigma Aldrich and were freshly diluted in isotonic saline (0.9% NaCl). The injection volume was always 0.1 mL/10 g body weight. The schedule of drug treatment and behavioral tasks is illustrated in fig.1A.

2.1. Hemiparkinsonism, dyskinesia and exercise

We used C57BL/6 mice (male, 8-10 weeks old, weighted 20-35 g) from our own inbred colony, housed on a 12/12-h light/dark cycle (lights

ON 7 A.M.), room temperature $21\pm 1^{\circ}\text{C}$, with *ad libitum* access to food and water.

6-Hydroxydopamine hydrochloride (6-OHDA, 3 μg in 1 μL of 0.1% sodium metabisulfite diluted in 0.9% NaCl) was injected via stereotactic surgery, under deep anesthesia (tribromoethanol 300 mg/kg, i.p.), in two different regions of the right mid-striatum ($2 \times 2 \mu\text{L}$, 0.5 $\mu\text{L}/\text{min}$), with the following coordinates in mm (Santini et al., 2007): (i) AP +1, ML -2.1, DV -3.2; and (ii) AP +0.3, ML -2.3, DV -3.2.

Not all Parkinsonian animals develop LID (Cenci, 2007, Santini et al., 2007). Then we selected animals with severe hemiparkinsonism, which increases the risk of development of LID (Santini et al., 2007). Mice were challenged with R(-)-apomorphine (0.6 mg/kg, s.c.), and after cylinder habituation (10 min), the number of net rotations were evaluated in plastic tubes (19 cm diameter, 22 cm high, 30 min) using the video tracking system ANY-maze™ (Stoelting, USA). After four weeks of recovery from 6-OHDA surgery, those animals with insufficient number of net rotations (> 2 counterclockwise rotations/min, fig.1B) (Nishimura et al., 2003) were discarded (around 20-30%/experiment).

Selected mice were then individually housed in cages ($28 \times 17 \times 13$ cm) with free access either to running wheels (exercise group) or to locked versions of the same wheels (sedentary group). The

RW (RW 4½'', Super Pet, USA) were fitted with electronic counters for distance and speed (Cunha et al., 2012). The locked wheels were used to avoid bias of environmental enrichment (EE) due to the introduction of objects (wheels) into mouse cages. Moreover, all wheels remained at the same fixed position in the cages to avoid spatial EE.

In parallel to the exposure to the wheels (fig.1A), all sedentary and exercised mice were daily treated with a single intraperitoneal (i.p.) injection of L-DOPA methyl ester hydrochloride (25 mg/kg) (Pavon et al., 2006) and benserazide hydrochloride (12.5 mg/kg), a peripheral DOPA decarboxylase inhibitor. L-DOPA/benserazide treatment and exposure to the wheels lasted two weeks. This protocol yielded the two experimental groups of interest, namely hemiparkinsonian (6-OHDA-treated mice exposed to L-DOPA) that were either (i) sedentary (SED, with locked wheel, $n = 31$) or (ii) exercised (RW, with unlocked running wheel, $n = 28$).

2.2. Behavioral tasks

Animals were habituated for 1 h in a sound and light (12 lx) attenuated room before behavioral tasks, which were carried out during the light phase of the cycle (10-17 PM).

Cylinder task was assessed in individual glass cylinders (12 cm diameter, 15 cm high) at three different times: (i) before 6-OHDA lesion, (ii) four weeks after 6-OHDA surgery and before L-DOPA/benserazide treatment, and (iii) two weeks after L-DOPA/benserazide treatment (twenty minutes after injection). We manually evaluated the number of forepaw contacts with the cylinder walls during 3 min. We only counted paw contacts with extended digits supporting the body weight. Results were expressed as the number of failures of injured paw (left paw, right striatum) as a % of total number of wall contacts, which represents an asymmetry score (contralateral bias) (Pavon et al., 2006).

Abnormal involuntary movements (AIMs) were assessed in larger plastic tubes (19 cm diameter, 22 cm high) over a period of 120 min after two weeks of L-DOPA/benserazide treatment. AIMs were manually classified into: orofacial, forelimb, axial or dystonic, and locomotive (Video 1); and severity was scored for 1 min every 20 min using a 0-4 scale as previously defined (Lundblad et al., 2005, Pavon et al., 2006). The video tracking system ANY-maze™ (Stoelting, USA) was used for off-line rating of locomotive AIMs (rotations), where we also scored rearing and grooming, as conventional measures of motor activity. When testing the only approved antidyskinetic drug (Lundblad et al., 2005), Amantadine

hydrochloride (60 mg/kg, i.p.) was administered 90 min before L-DOPA/benserazide injection.

2.3. Biochemistry

Forty min after the last L-DOPA/benserazide injection, striata and quadriceps muscle samples were dissected and freshly processed according to the procedures described below. Mice were also anesthetized (Pentobarbital 50 mg/kg, i.p.) and intracardially perfused with 4% paraformaldehyde (PFA) in phosphate buffer (pH 7.4). Brains were post-fixed with 4% PFA (2 h) and 25% sucrose (24 h).

Muscle mitochondrial activity was assessed by enzymatic activity of complex I enzyme corrected by citrate synthase (CS), or complex I/CS rate. Quadriceps femoris muscle was mechanically homogenized in 50 mM of phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.46 mM KH₂PO₄, pH 7.4) containing 0.3 M saccharose, 5 mM 3-(N-morpholino)-propanesulfonic sodium salt (MOPS), 1 mM EGTA, and 0.1% bovine serum albumin. The homogenates were centrifuged (1000 × g, 10 min, 4°C) and supernatant was centrifuged at 15000 × g in order to concentrate mitochondria in the pellet, which was dissolved in the same phosphate buffer (Latini et al., 2005). We then measured CS and complex I in mitochondria-enriched fractions (2 ml/mg protein) (Lowry et al., 1951).

Complex I activity was measured through ferricyanide reduction ($\epsilon = 1 \text{ mM}^{-1} \times \text{cm}^{-1}$, 30°C) at 420 nm (Cassina and Radi, 1996). CS activity was measured at 420 nm in 1 M potassium phosphate buffer (1 M KH_2PO_4 and 1 M K_2PO_4 , pH 7.4) containing 0.5 mM oxaloacetate, 0.31 mM acetyl-CoA, 0.1 mM 5,5-dithiobis(2-nitrobenzoic acid), and 0.25% Triton X-100. Complex I and CS were calculated as nmol/min/mg protein.

Immunohistochemistry (IHC). Protein extracts from striata were prepared as described (Aguiar et al., 2010) and proteins (20 μg) (Lowry et al., 1951) were separated by 10% SDS-PAGE gel electrophoresis and blotted onto a nitrocellulose membrane. Immunodetection was carried out using different polyclonal primary antibodies, namely rabbit anti- DARPP-32 total, phospho-Thr(75) and phospho-Thr(34) (1:1000, Cell Signaling[®]), rabbit anti- c-Fos (1:200, Merck[®]), or mouse anti- cyclin-dependent kinase 5 (cdk 5) (1:200, Santa Cruz[®]). Immunoreactivity was visualized by enhanced chemiluminescence and quantified using ImageJ platform (1.44p, National Institute of Health). The IHC detection of tyrosine hydroxylase (TH), was carried out in free-floating coronal striatal sections (25 μm thick) using a standard avidin-biotin IHC protocol with a monoclonal primary antibody against TH (1:1000, IgG, Diasorin) (Prediger et al., 2010) and the IHC quantification was also quantified using the ImageJ platform.

High performance liquid chromatography (HPLC). Blood was collected immediately after anesthesia (Pentobarbital 50 mg/kg, i.p.) into an EDTA solution to allow the separation of plasma by centrifugation. The plasma was then neutralized (2 M $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, pH 7.6) and re-centrifuged ($30000 \times g$, 15 min, 4°C). Aliquots (20 μl) were analyzed by reverse phase HPLC (Ultrasphere IP with particle size of 5 μm), using electrochemical detection, as previously described (Prediger et al., 2010).

2.4. Statistical analysis

Data are presented as mean \pm standard error of the mean (SEM). The contralateral bias (%) has been transformed (cosine transform) to allow parametric statistical comparison. Student's *t*-test, analysis of variance (ANOVA) and Bonferroni *post-hoc* test were used for comparisons of the means. The relation between AIMS and cdk5 was performed by Pearson's linear regression. The accepted level of significance was $p < 0.05$.

3. RESULTS

Hemiparkinsonian mice allowed to freely exercise (RW) run maximal distances close to 1.5 km/day (fig.1C). These animals also displayed muscular adaptations to exercise training (Aguiar et al., 2008b), typified by a $17.7 \pm 4.2\%$ increment ($p < 0.05$) of complex I/CS rate in the

skeletal muscle (462.3 ± 18.4) when compared to sedentary (402.4 ± 10.4); accordingly, during the weeks of exercise, these hemiparkinsonian mice doubled their average running speed and increased fourfold their maximum running speed ($p < 0.05$, fig.1D). These results demonstrate the increased muscle oxidative activity and exercise performance after 14 days of RW.

3.1. Repeated L-DOPA administration improves 6-OHDA-induced motor deficits, but induces dyskinesia

The hemiparkinsonian state of the 6-OHDA-treated mice was first confirmed using two different criteria: 1) first, while alive, mice were challenged with R(-)-apomorphine; 2) second, after being killed, we carried out an IHC analysis of striatal TH. As expected, hemiparkinsonian mice displayed a marked increase of R(-)-apomorphine-induced counterclockwise rotations in comparison to control (vehicle-injected) mice ($p < 0.05$, fig.1B). Also, 6-OHDA also caused a significant $83 \pm 4\%$ decrease ($p < 0.05$) of the striatal TH immunostaining in the injected striatum in relation to contralateral unlesioned hemisphere, which was similar in sedentary (SED) (fig.2A) and exercised (RW) mice (fig.2B).

We also confirmed that 6-OHDA-induced hemiparkinsonism was accompanied by a significative bradykinesia in the left forelimb, which was attenuated after L-DOPA/benserazide treatment ($p < 0.05$, fig.2C).

Additionally, the long-term treatment with L-DOPA induced the development of AIMs (fig.2D-F), as testified by the ability of Amantadine (60 mg/kg, i.p) to decrease the severity of LID ($p < 0.05$, fig.2D), thus confirming the dyskinetic phenotype of these abnormal movements.

3.2. Exercise selectively reduces the development of L-DOPA-induced dyskinesia

Exercise and L-DOPA/benserazide did not affect 6-OHDA-induced neurodegeneration, since SED and RW mice showed similar loss of TH immunoreactivity (sedentary = 0.16 ± 0.8 , RW = 0.18 ± 0.7 , arbitrary unities) in the striatum compared to the contralateral hemisphere (sedentary = 0.98 ± 0.6 , RW = 0.96 ± 0.8 , arbitrary unities) (fig.2A-B). Also, another important factor predisposing to the development of LID, i.e. the plasma levels of L-DOPA (Lundblad et al., 2005, Cenci, 2007), was unaffected by exercise (SED 21.3 ± 1.4 , RW 22.4 ± 2.4 , $\mu\text{g/mL/kg}$ body weight). Notably, hemiparkinsonian mice allowed to freely exercise presented less severe L-DOPA-induced axial and forelimb AIMs ($p < 0.05$, fig.2D-E) and less stereotyped rotations than SED animals ($p < 0.05$, fig.2F). However, the orofacial AIMs were not modified by exercise (fig.2E). Moreover, the discrete EE of locked wheels, however, did not alter the development of LID in SED animals (fig.2D-F).

These antidyskinetic effects were not associated with differences in overall motor function, since spontaneous locomotion was unaffected by exercise; in fact, rearing (SED 4 ± 0.6 , RW, 3.3 ± 0.4) and grooming (SED 6.5 ± 0.4 , RW 6.2 ± 0.4) activities in the cylinder, as well as the distance travelled in the tube (SED 33 ± 5.3 m, RW 27.4 ± 4.2 m), were similar between SED and RW groups.

3.3. Exercise attenuates striatal neurochemical changes associated to L-DOPA-induced dyskinesia

Cdk5 and DARPP-32 abnormal signaling is tightly associated with the phenotype of LID in murine (Andersson et al., 1999, Santini et al., 2007). Accordingly, we confirmed a significant correlation between the severity of LID and the striatal levels of cdk5 ($p < 0.05$, fig.3A), which was increased in the lesioned striatum of SED (1.5 ± 0.1 arbitrary units, a.u.). Notably the striatal cdk5 immunoreactivity of the lesioned striatum was reversed by exercise (0.9 ± 0.1 a.u. in RW mice) to similar levels to that found in the contralateral unlesioned hemisphere (SED 1 ± 0.2 , RW 1 ± 0.2).

Chronic treatment with L-DOPA also increased the phosphorylation of Thr(34) and Thr(75) sites of DARPP-32 protein in the lesioned striatum of SED ($p < 0.05$, fig.3C); notably, this was restricted to Thr(75) site of

DARPP-32 in exercised mice ($p < 0.05$, fig.3C). Thus, exercise induced a significant decrease of Thr75/Thr34 phosphorylation ratio in the dyskinetic animals (SED 1.46 ± 0.05 , RW 1.19 ± 0.05 , $p < 0.05$).

Finally, we also confirmed that L-DOPA treatment increased c-Fos expression, a biomarker of neuronal activity (Liste et al., 1997), in the lesioned striatum of dyskinetic animals ($p < 0.05$, fig.3B). Notably, c-Fos levels were significantly lower in the lesioned striatum of hemiparkinsonian mice allowed to exercise (RW) in relation to SED ($p < 0.05$, fig.3).

4. DISCUSSION

The present study shows that exercise attenuates the development of LID, the main side-effect and limiting factor associated with the therapeutic management of PD (Parkes, 1981, Maurel et al., 2001). Furthermore, we provide evidence that this beneficial effect of exercise results from its ability to curtail the aberrant dopaminergic signaling in the striatum that is currently accepted to underlie LID (Andersson et al., 1999, Picconi et al., 2003, Santini et al., 2007). The appearance of dyskinesia represents a challenge to PD therapy because it can be severe enough to warrant reducing the L-DOPA dose below optimal therapeutic levels (Pavon et al., 2006). Interesting, earlier evidence had demonstrated increased efficiency

of suboptimal doses of L-DOPA in PD patients and MPTP-treated mice (Archer and Fredriksson, 2010, Frazzitta et al., 2012). These patients had milder dyskinesia, without any increase in motor symptoms (Frazzitta et al., 2012). Our results extend these findings by demonstrating that exercise can attenuate the development of LID in optimal doses of the drug.

Evidence have consistently demonstrated benefits of exercise to brain (Cotman and Berchtold, 2002, Aguiar et al., 2008a), in particular to attenuate the burden of brain diseases, including PD (Petzinger et al., 2007, Teixeira et al., 2008, Aguiar et al., 2009). However, wheels also allow any exploration and interaction beyond running, such as climbing, chewing and digging, a form of EE. The literature shows several profiles of neuroplasticity in murine exposed to EE (Cotman and Berchtold, 2002). However, some authors suggest diminishing neuroplasticity changes in the absence of RW on the EE (van Praag et al., 2000). Our results are consistent with this evidence. In this work, the running motion was exclusive to RW group, as described in the Methods and Results section. Neurological benefits of exercise may also result from increased motor skills (McCloskey et al., 2001, Aguiar et al., 2009). Accordingly, we now report that exercised hemiparkinsonian animals ran more (increasing distances raced) and faster (increasing running speeds) than sedentary hemiparkinsonian controls (locked wheels), which is indicative of

increased athletic performance and motor control, respectively. Biomechanical analysis revealed a fine trunk and limbs coordination during these rapid movements (Walter, 2003); it was precisely trunk and limbs that benefited from the antidyskinetic effects of exercise, instead of face and tongue, which control is distributed through different corticostriatal loops (Holschneider et al., 2007). Furthermore, some authors have reported an increased efficacy of L-DOPA (Archer and Fredriksson, 2010, Ung et al., 2012), including in patients with PD (Reuter et al., 1999, Muhlack et al., 2007, Frazzitta et al., 2012), which could explain the beneficial effects of exercise. Moreover, our findings indicate that exercise did not affect the antiparkinsonian effects of L-DOPA in hemiparkinsonian animals, nor did it modify the plasma levels of L-DOPA in accordance with the lack of impact of exercise on the pharmacokinetics and pharmacodynamics of L-DOPA in PD patients (Muhlack et al., 2007, Lopane et al., 2010). This supports the hypothesis that exercise may selectively rebalance the functioning of the basal ganglia to permit L-DOPA to partially restore dopaminergic tonus without development of LID. This contention is further supported by the ability of exercise to prevent the development of the cardinal neurochemical changes in the basal ganglia that are known to be associated with LID (Liste et al., 1997, Aguiar et al., 2010, VanLeeuwen et al., 2010, Aguiar Jr et al., 2012).

LID is associated with some characteristic neurochemical changes in the basal ganglia: it is dependent on the over-functioning of DA D₁-like receptors in the striatopallidal MSNs (Aubert et al., 2005), leading to increased phosphorylation at threonine 34 of DARPP-32 and inhibition of PP-1 (Santini et al., 2007); this results in a loss of depotentiation and enhanced activation of the striatopallidal neurons (Picconi et al., 2003), typified increased FosB expression (Pavon et al., 2006), which is currently considered the main neurophysiologic trait associated with LID in animal models of PD (Picconi et al., 2003, Santini et al., 2007). Active DARPP-32-Thr(34) enhances phosphorylation of NMDA receptors, which provides a rationale to understand the ability of Amantadine to achieve its long-term antidyskinetic effects, albeit its clinical use is limited by the side effects resulting from its interference with all forms of plasticity dependent on NMDA receptors (Lundblad et al., 2005). Further adding to this dyskinesia-associated unbalanced regulation of DARPP-32, a key integrator of the responsiveness of MSNs (Picconi et al., 2003), dyskinetic animals also present higher levels of cdk5 in the striatum (Aubert et al., 2005), which phosphorylates DARPP-32 at threonine 75 and converts DARPP-32 into an inhibitor of protein kinase A (PKA) (Picconi et al., 2003, Santini et al., 2007). The pharmacological and genetic manipulations to reduce the phosphorylation of DARPP-32 or PKA activity were also

successful in alleviating dyskinesia in hemiparkinsonian animals (Santini et al., 2007, Darmopil et al., 2009, Lebel et al., 2010). We now report that the dyskinetic mice able to self-exercise displayed a reduction of some of the key neurochemical features associated with dyskinesia, namely decreased Fos expression, decreased DARPP-32 phosphorylation and decreased cdk5 activation in the striatum. Thus, voluntary exercise curtailed the activation of DARPP-32 at threonine 34. Furthermore, it decreased cdk5 activation and caused an even greater decrease of the phosphorylation pattern of DARPP-32 at threonine 75. This resulted in an expected decreased activation of the basal ganglia circuitry, as gauged by the decreased up-regulation of c-Fos in dyskinetic mice able to exercise. Altogether, these observations further strengthen our contention that exercise is able to rebalance dopaminergic over-signaling in the striatum, thus curtailing the severity of LID.

This key ability of exercise to normalize the over-sensitive dopaminergic signaling in MSNs is in notable agreement with evidence showing the ability of exercise to counteract drug-induced stereotyped behaviors affecting dopaminergic system, such as haloperidol-induced catalepsy (Klusha et al., 1983), apomorphine-induced rotations (Aguiar Jr et al., 2012), or yawning movements induced by either apomorphine (Dey and Singh, 1992) or reserpine (Teixeira et al., 2008). In all these abnormal

movements, exercise counteracted an aberrant functioning of the dopaminergic signalling in the basal ganglia. Future work ought to be undertaken to tackle the mechanisms by which exercise exerts this normalizing effect. A tentative possibility would be an ability of exercise to bolster the regeneration of the dopaminergic system blunted in PD, given that the presynaptic dopaminergic robustness in the striatum determines the susceptibility to LID (Picconi et al., 2003, Cenci, 2007). This hypothesis is heralded by the observations that exercise enhances the survival of dopaminergic neurons and increases neurogenesis in the striatum of animal models of PD (O'Dell et al., 2007, Yoon et al., 2007). This would be a tight analogy with the ability of exercise to attenuate memory impairment in Parkinsonism (Aguilar et al., 2009), which involves a growth factor mediated (reviewed in Cotman and Berchtold, 2002). An alternative hypothesis would be an impact of exercise on the glutamatergic afferents driving the basal ganglia, in accordance with the impact of exercise on the morpho-functional plasticity of glutamatergic synapses (Real et al., 2010, VanLeeuwen et al., 2010) that are known to be affected in dyskinetic animals (Kobylecki et al., 2010, Sgambato-Faure and Cenci, 2012).

This association (L-DOPA plus exercise) is safe and used since the introduction of L-DOPA forty years ago (Blonsky and Minnigh, 1970, Mikkelsen et al., 1972). We suggest that exercise could interfere with

development of dyskinesia and abnormal striatal signaling after L-DOPA treatment. These findings can widen the symptomatic benefits of primary antiparkinsonian drug, L-DOPA. These data should be considered in future clinical studies involving PD, exercise and L-DOPA treatment.

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6. REFERENCES

Aguiar AS, Jr., Araujo AL, da-Cunha TR, Speck AE, Ignacio ZM, De-Mello N, Prediger RD (2009) Physical exercise improves motor and short-term social memory deficits in reserpinized rats. *Brain Res Bull* 79:452-457.

Aguiar AS, Jr., Boemer G, Rial D, Cordova FM, Mancini G, Walz R, de Bem AF, Latini A, Leal RB, Pinho RA, Prediger RD (2010) High-intensity physical exercise disrupts implicit memory in mice: involvement of the striatal glutathione antioxidant system and intracellular signaling. *Neuroscience* 171:1216-1227.

Aguiar AS, Jr., Speck AE, Prediger RD, Kapczinski F, Pinho RA (2008a) Downhill training upregulates mice hippocampal and striatal brain-derived neurotrophic factor levels. *J Neural Transm* 115:1251-1255.

Aguiar AS, Jr., Tuon T, Soares FS, da Rocha LG, Silveira PC, Pinho RA (2008b) The effect of n-acetylcysteine and deferoxamine on exercise-induced oxidative damage in striatum and hippocampus of mice. *Neurochem Res* 33:729-736.

Aguiar Jr AS, Boemer G, Duzzioni M, Raisman-Vozari R, Prediger RD (2012) 3.264 Further evidences of exercise-induced neuroprotection in 6-OHDA-hemiparkinsonian mice. *Parkinsonism & Related Disorders* 18, Supplement 2:S219.

Andersson M, Hilbertson A, Cenci MA (1999) Striatal fosB expression is causally linked with l-DOPA-induced abnormal involuntary movements and the associated upregulation of striatal prodynorphin mRNA in a rat model of Parkinson's disease. *Neurobiol Dis* 6:461-474.

Archer T, Fredriksson A (2010) Physical exercise attenuates MPTP-induced deficits in mice. *Neurotox Res* 18:313-327.

Aubert I, Guigoni C, Hakansson K, Li Q, Dovero S, Barthe N, Bioulac BH, Gross CE, Fisone G, Bloch B, Bezard E (2005) Increased D1 dopamine receptor signaling in levodopa-induced dyskinesia. *Ann Neurol* 57:17-26.

Blonsky ER, Minnigh EC (1970) The modifying influence of L-dopa on the physical therapy program in Parkinson's disease. *Prog Phys Ther* 1:55-74.

Cassina A, Radi R (1996) Differential inhibitory action of nitric oxide and peroxynitrite on mitochondrial electron transport. *Arch Biochem Biophys* 328:309-316.

Cenci MA (2007) Dopamine dysregulation of movement control in L-DOPA-induced dyskinesia. *Trends Neurosci* 30:236-243.

Cotman CW, Berchtold NC (2002) Exercise: a behavioral intervention to enhance brain health and plasticity. *Trends Neurosci* 25:295-301.

Cunha MP, Oliveira A, Pazini FL, Machado DG, Bettio LE, Budni J, Aguiar AS, Jr., Martins DF, Santos AR, Rodrigues AL (2012) The Antidepressant-like Effect of Physical Activity in the Voluntary Running Wheel. *Med Sci Sports Exerc*.

Darmopil S, Martin AB, De Diego IR, Ares S, Moratalla R (2009) Genetic inactivation of dopamine D1 but not D2 receptors inhibits L-DOPA-induced dyskinesia and histone activation. *Biol Psychiatry* 66:603-613.

Dey S, Singh RH (1992) Modification of apomorphine-induced behaviour following chronic swim exercise in rats. *Neuroreport* 3:497-500.

Frazzitta G, Bertotti G, Morelli M, Riboldazzi G, Pelosin E, Balbi P, Boveri N, Comi C, Turla M, Leva S, Felicetti G, Maestri R (2012)

Rehabilitation improves dyskinesias in Parkinsonian patients: a pilot study comparing two different rehabilitative treatments. *NeuroRehabilitation* 30:295-301.

Holschneider DP, Yang J, Guo Y, Maarek JM (2007) Reorganization of functional brain maps after exercise training: Importance of cerebellar-thalamic-cortical pathway. *Brain Res* 1184:96-107.

Klusha B, Georgiev V, Petkov V, Markovska V (1983) Effects of the tripeptides TRH, MIF and MAF on the cerebral dopaminergic system. *Acta Physiol Pharmacol Bulg* 9:14-19.

Kobylecki C, Cenci MA, Crossman AR, Ravenscroft P (2010) Calcium-permeable AMPA receptors are involved in the induction and expression of l-DOPA-induced dyskinesia in Parkinson's disease. *J Neurochem* 114:499-511.

Latini A, Rodriguez M, Borba Rosa R, Scussiato K, Leipnitz G, Reis de Assis D, da Costa Ferreira G, Funchal C, Jacques-Silva MC, Buzin L, Giugliani R, Cassina A, Radi R, Wajner M (2005) 3-Hydroxyglutaric acid moderately impairs energy metabolism in brain of young rats. *Neuroscience* 135:111-120.

Lebel M, Chagniel L, Bureau G, Cyr M (2010) Striatal inhibition of PKA prevents levodopa-induced behavioural and molecular changes in the hemiparkinsonian rat. *Neurobiol Dis* 38:59-67.

Liste I, Guerra MJ, Caruncho HJ, Labandeira-Garcia JL (1997) Treadmill running induces striatal Fos expression via NMDA glutamate and dopamine receptors. *Exp Brain Res* 115:458-468.

Lopane G, Contin M, Scaglione C, Albani F, Baruzzi A, Martinelli P (2010) The effect of a clinically practical exercise on levodopa bioavailability and motor response in patients with Parkinson disease. *Clin Neuropharmacol* 33:254-256.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265-275.

Lundblad M, Usiello A, Carta M, Hakansson K, Fisone G, Cenci MA (2005) Pharmacological validation of a mouse model of l-DOPA-induced dyskinesia. *Exp Neurol* 194:66-75.

Maurel F, Lilliu H, Le Pen C (2001) [Social and economic cost of L-Dopa-induced dyskinesias in patients with Parkinson's disease]. *Rev Neurol (Paris)* 157:507-514.

McCloskey DP, Adamo DS, Anderson BJ (2001) Exercise increases metabolic capacity in the motor cortex and striatum, but not in the hippocampus. *Brain Res* 891:168-175.

Mikkelsen B, Dupont E, Worm-Petersen J (1972) The influence of L-dopa on physical treatment of Parkinsonism. *Acta Neurol Scand Suppl* 51:127-128.

Muhlack S, Welnic J, Woitalla D, Muller T (2007) Exercise improves efficacy of levodopa in patients with Parkinson's disease. *Mov Disord* 22:427-430.

Nishimura F, Yoshikawa M, Kanda S, Nonaka M, Yokota H, Shiroi A, Nakase H, Hirabayashi H, Ouji Y, Birumachi J, Ishizaka S, Sakaki T (2003) Potential use of embryonic stem cells for the treatment of mouse parkinsonian models: improved behavior by transplantation of in vitro differentiated dopaminergic neurons from embryonic stem cells. *Stem Cells* 21:171-180.

O'Dell SJ, Gross NB, Fricks AN, Casiano BD, Nguyen TB, Marshall JF (2007) Running wheel exercise enhances recovery from nigrostriatal dopamine injury without inducing neuroprotection. *Neuroscience* 144:1141-1151.

Parkes JD (1981) Adverse effects of antiparkinsonian drugs. *Drugs* 21:341-353.

Pavon N, Martin AB, Mendiola A, Moratalla R (2006) ERK phosphorylation and FosB expression are associated with L-DOPA-induced dyskinesia in hemiparkinsonian mice. *Biol Psychiatry* 59:64-74.

Petzinger GM, Walsh JP, Akopian G, Hogg E, Abernathy A, Arevalo P, Turnquist P, Vuckovic M, Fisher BE, Togasaki DM, Jakowec MW (2007) Effects of treadmill exercise on dopaminergic transmission in the 1-methyl-

4-phenyl-1,2,3,6-tetrahydropyridine-lesioned mouse model of basal ganglia injury. *J Neurosci* 27:5291-5300.

Picconi B, Centonze D, Hakansson K, Bernardi G, Greengard P, Fisone G, Cenci MA, Calabresi P (2003) Loss of bidirectional striatal synaptic plasticity in L-DOPA-induced dyskinesia. *Nat Neurosci* 6:501-506.

Prediger RD, Aguiar AS, Jr., Rojas-Mayorquin AE, Figueiredo CP, Matheus FC, Ginestet L, Chevarin C, Bel ED, Mongeau R, Hamon M, Lanfumey L, Raisman-Vozari R (2010) Single intranasal administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in C57BL/6 mice models early preclinical phase of Parkinson's disease. *Neurotox Res* 17:114-129.

Real CC, Ferreira AF, Hernandez MS, Britto LR, Pires RS (2010) Exercise-induced plasticity of AMPA-type glutamate receptor subunits in the rat brain. *Brain Res* 1363:63-71.

Reuter I, Engelhardt M, Stecker K, Baas H (1999) Therapeutic value of exercise training in Parkinson's disease. *Med Sci Sport Exer* 31:1544-1549.

Santini E, Valjent E, Usiello A, Carta M, Borgkvist A, Girault JA, Herve D, Greengard P, Fisone G (2007) Critical involvement of cAMP/DARPP-32 and extracellular signal-regulated protein kinase signaling in L-DOPA-induced dyskinesia. *J Neurosci* 27:6995-7005.

Sgambato-Faure V, Cenci MA (2012) Glutamatergic mechanisms in the dyskinesias induced by pharmacological dopamine replacement and deep

brain stimulation for the treatment of Parkinson's disease. *Prog Neurobiol* 96:69-86.

Teixeira AM, Trevizol F, Colpo G, Garcia SC, Charao M, Pereira RP, Fachineto R, Rocha JB, Burger ME (2008) Influence of chronic exercise on reserpine-induced oxidative stress in rats: behavioral and antioxidant evaluations. *Pharmacol Biochem Behav* 88:465-472.

Ung RV, Rouleau P, Guertin PA (2012) Functional and physiological effects of treadmill training induced by buspirone, carbidopa, and L-DOPA in clenbuterol-treated paraplegic mice. *Neurorehabil Neural Repair* 26:385-394.

van Praag H, Kempermann G, Gage FH (2000) Neural consequences of environmental enrichment. *Nat Rev Neurosci* 1:191-198.

VanLeeuwen JE, Petzinger GM, Walsh JP, Akopian GK, Vuckovic M, Jakowec MW (2010) Altered AMPA receptor expression with treadmill exercise in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned mouse model of basal ganglia injury. *J Neurosci Res* 88:650-668.

Walter RM (2003) Kinematics of 90 degrees running turns in wild mice. *J Exp Biol* 206:1739-1749.

Yoon MC, Shin MS, Kim TS, Kim BK, Ko IG, Sung YH, Kim SE, Lee HH, Kim YP, Kim CJ (2007) Treadmill exercise suppresses nigrostriatal

dopaminergic neuronal loss in 6-hydroxydopamine-induced Parkinson's rats. *Neurosci Lett* 423:12-17.

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Video and figure legends

Video 1. L-DOPA-induced dyskinesia.

Figure 1. (Panel A) Experimental design: all animals received a double intra-striatal administration of 6-OHDA (0 week) and experienced a typical hemiparkinsonism phenotype, revealed by increased counterclockwise rotations (4 weeks) ($p < 0.05$ vs. vehicle, Student's *t*-test, panel B). Mice were then separated in exercise (running wheels) or sedentary groups, and were simultaneously treated with L-DOPA/benserazide (4-6 weeks). Exercised mice improved motor skills, as showed by daily increased running distances and speeds (repeated measures ANOVA followed by Bonferroni *post-hoc* test, panels C and D). Data represent mean \pm SEM for three independent experiments ($n = 9-11$ animals/group/experiment). * $p < 0.05$ versus vehicle (Student's *t*-test). Abbreviations: R(-)-APO – R(-)-apomorphine, 6-OHDA – 6-hydroxydopamine.

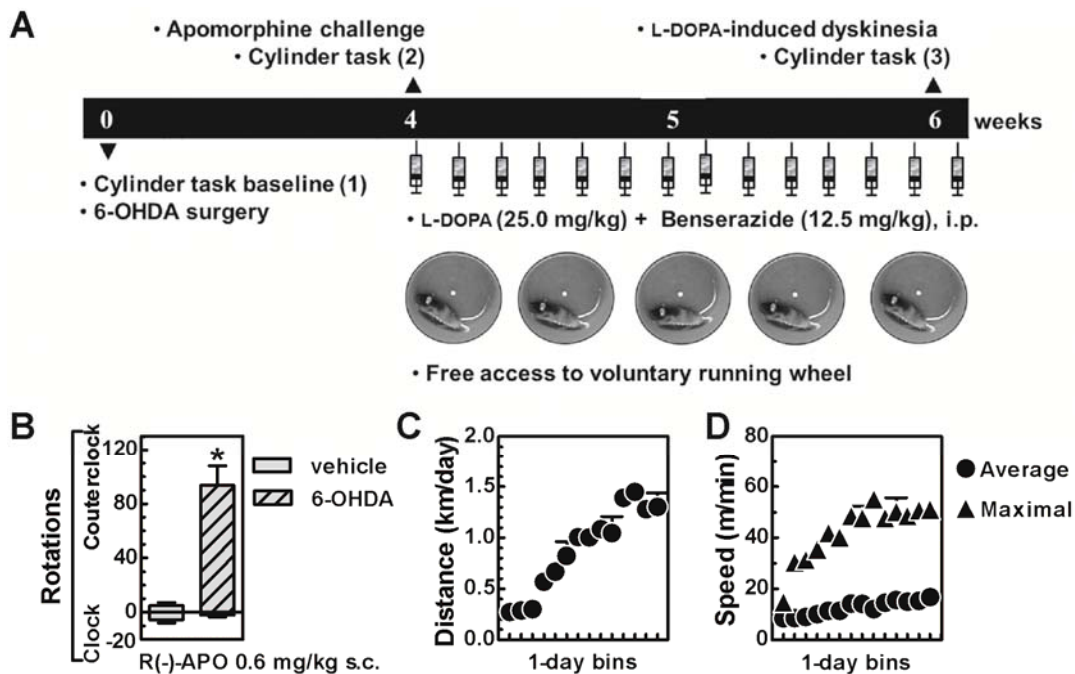
Figure 2. Exercise and L-DOPA/benserazide (25/12.5 mg/kg, i.p.) showed no effect on neurodegeneration, evaluated as a decreased immunoreactivity of tyrosine hydroxylase in the lesioned striatum (A-B). Exercise also not changed the antiparkinsonian effects of L-DOPA ($p < 0.05$ vs. baseline, # $p < 0.05$ vs. 4 weeks, repeated measures ANOVA followed by Bonferroni

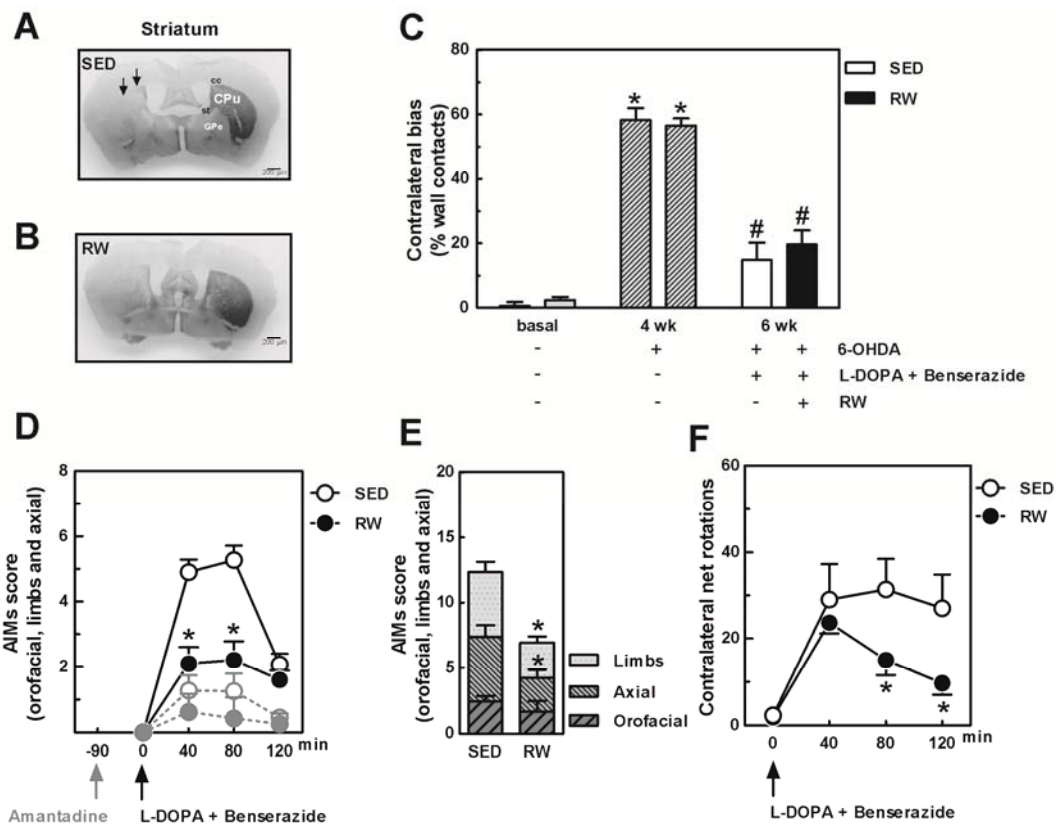
post-hoc test, panel C). However, exercise effective in reducing LID ($p < 0.05$ vs. SED, repeated measures ANOVA followed by Bonferroni *post-hoc* test, panels D and F), markedly for the AIMs of the limbs and trunk, but not orofacial ($p < 0.05$, Student's *t*-test, panel E). Data represent mean \pm SEM for three independent experiments ($n = 9-11$ animals/group/experiment). Abbreviations: AIMs – abnormal involuntary movements, CPu – Caudate-putamen, GPe – external globus pallidus, RW – running wheel, sc – corpus callosum, SED – sedentary, st – stria terminalis, 6-OHDA – 6-hydroxydopamine.

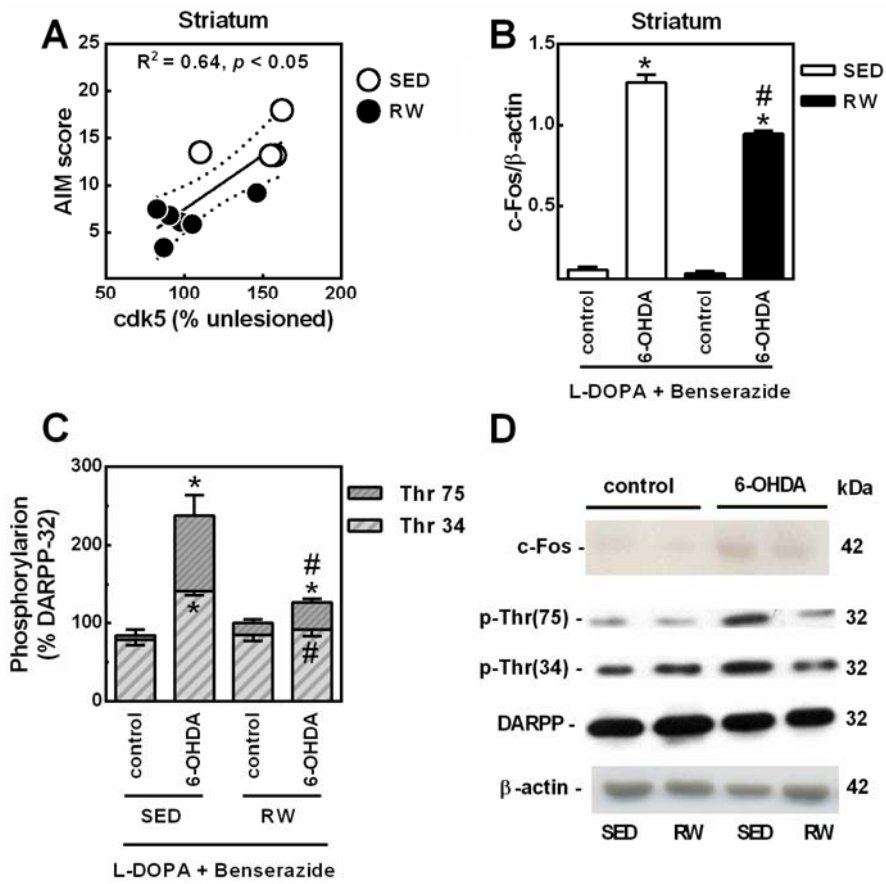
Figure 3. The severity of L-DOPA-induced dyskinesia correlated with increased striatal levels of cdk5 (Pearson's correlation, panel A) and c-Fos ($p < 0.05$ vs. control, # $p < 0.05$ vs. 6-OHDA, two-way ANOVA followed by Bonferroni *post-hoc* test, panel B), an indicative of enhanced neuronal activity. L-DOPA also triggered an hyperphosphorylation of DARPP-32 at Thr(34) and Thr(75) sites in sedentary mice ($p < 0.05$ vs. control, # $p < 0.05$ vs. 6-OHDA, two-way ANOVA followed by Bonferroni *post-hoc* test, panel C). Notably, exercise (RW) decreased these neurochemical changes typically associated with L-DOPA-induced dyskinesia. Data represent mean \pm SEM for two independent experiments ($n = 6-8$ animals/group/experiment). AIM – abnormal involuntary

movements, cdk5 – cyclin-dependent kinase 5, DARPP-32 – dopamine and cAMP-Regulated Phosphoprotein 32 kDa, RW – running wheel, SED – sedentary, Thr – threonine residue, 6-OHDA – 6-hydroxydopamine.

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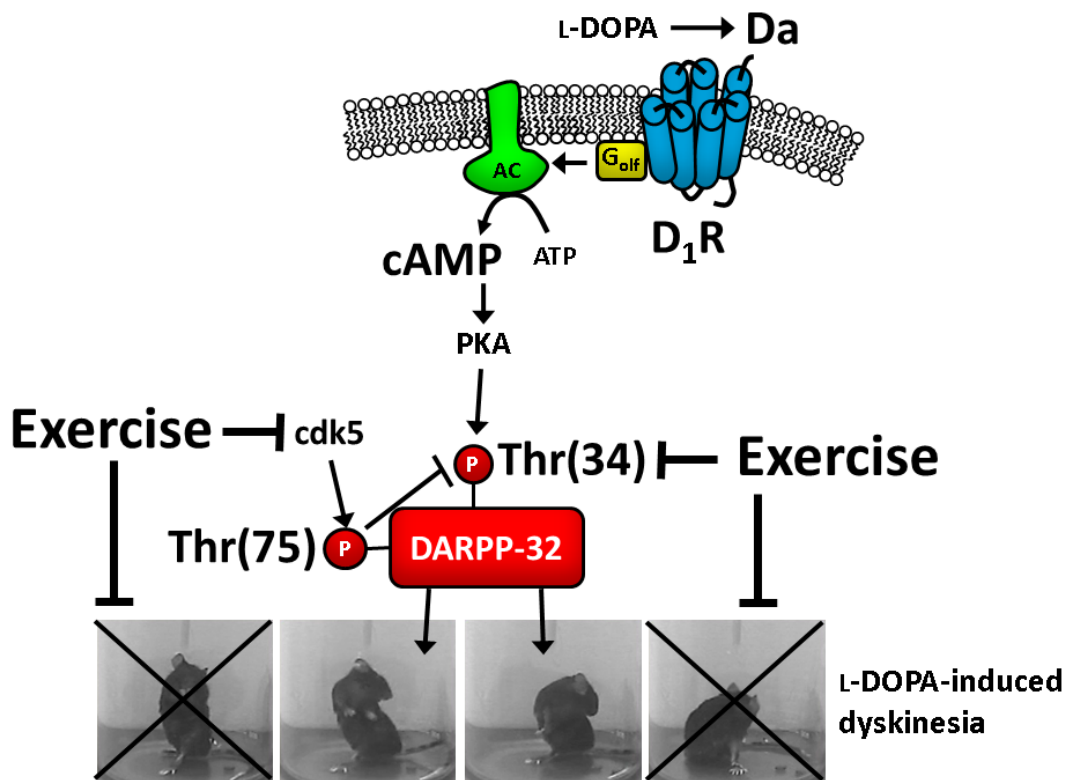






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Long-term L-DOPA induced dyskinesia in hemiparkinsonian animals

Voluntary exercise showed antidyskinetic effects

The abnormal movements of the trunk and limbs were attenuated, but not orofacial

Exercise prevented abnormal dopaminergic signaling in the striatum of dyskinetic mice

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