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Amelioration of MPTP-toxicity by Running Exercise: Co-administration with Milmed.

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Abstract

Physical exercise, implying any and all activity that generates a force, through muscular activity, that disrupts a homeostatic state, holds inestimable benefits for general measures of function, quality-of-life, physical strength and endurance. It offers a nonpharmacologic, noninvasive intervention with manifest advantages for cerebral integrity during aging, enhancing brain health and plasticity. In the present study, applying the MPTP mouse model of Parkinsonism, physical exercise was combined with the electromagnetic wavelength-treated, *saccharomyces cerevisiae* yeast species, Milmed, to abolish/attenuate the marked hypokinesia induced by severe dopamine (DA) depletion over successive weeks, as well as the severe loss of DA in the striatal region of the C57/BL6 mice that were studied. The Milmed-exercise combination influenced markedly the capacity of groups of mice to exercise, as assessed during 30-min tests. It provides novel information pertaining to notions of pharmacogenomics, epigenetics, and biomarkers that are modulated by factors affecting the expression of disorder and possible therapeutic strategies.

Key-words: Parkinsonism – hypokinesia – dopamine – MPTP – exercise – Milmed – restoration – C57BL6 mice.

Introduction

It has been well-established that physical exercise both improves motor functioning and ameliorates biomarker deficits in several neuropsychiatric and neurodegenerative conditions (Archer, 2011, 2012; Archer and Kostrzewa, 2012; Archer et al., 2011a, b, 2012; Döbrössy and Nikkhah, 2012). Both clinical and laboratory evidence support the contention that exercise/activity attenuates the neurodegenerative process in Parkinson's disease (PD), as exemplified by motor performance and dopamine (DA) integrity (Archer and Fredriksson, 2010; 2012; 2013a, b; Fredriksson et al., 2011; Zigmond et al., 2009, 2012). It has been observed, for example, that motor exercise training facilitated self-repair following unilateral striatal lesions in rats (Döbrössy and Dunnett, 2003). Several lines of evidence have indicated that genetic, environmental and neuroimmunological factors are associated with the onset and progression of disorder (Hardy et al., 2006; Holford, 2013; Lazzarini et al., 2013; Moon et al., 2009), although the evaluation of risk factors remains uncertain (McGhee et al., 2013). Inflammatory processes contribute pathogenic factors accelerating both onset and progression of PD thereby underlining the links between damaged dopaminergic neurons and microglia (Ghosh et al., 2007; Kim and Joh, 2006; Roy et al., 2012). The influence of physical exercise upon instances of brain inflammation have accumulated; for example, it has been found to ameliorate the delayed secondary biochemical and cellular changes, associated with chronic neuroinflammation and progressive neurodegeneration, after traumatic brain injury that may continue for months or even years (Piao et al., 2013). Lipopolysaccharide-induced brain inflammation disturbs neuronal maturation forced treadmill exercise and voluntary wheel exercise improved brain inflammation-induced short-term memory impairment by suppressing doublecortin expression and increasing neuronal nuclear antigen expression thereby enhancing neuronal maturation (Kim et al., 2012).

Several epidemiological, genetic, pharmacological, and imaging lines of evidence support the proposal that inflammatory processes in this specific brain region are crucial for disease progression in PD (Tufekci et al., 2012). In rats subjected to chronic, mild unpredictable stress, swimming exercise reduced proinflammatory cytokines and serum corticosterone, increased serotonin levels and reduced depression symptoms (Liu et al., 2013); it seems possible that exercise may contribute to an anti-parkinsonism agency through an anti-inflammatory influence. It is currently considered to be at the forefront of interventions for PD (Earhart and Falvo, 2013; Grazina and Massaro, 2013). In this regard, Sung et al. (2012)

have shown that treadmill exercise prevented dopaminergic neuron loss, induced by MPTP, through the inhibition of brain inflammation by suppression of microglial activation in the PD mice. Resistence training has been shown to increase fat-free mass, muscle strength and endurance, in addition to noteworthy improvements in mobility and the performance of functional tasks by PD patients in randomized and nonrandomized controlled trial studies (Brienesse and Emerson, 3013).

Multiple doses of MPTP, 20-40 mg/kg, induce irreversible PD-like symptoms and loss of DA in human and non-human primates (Jackson-Lewis et al., 1995; Jonsson et al., 1985; Langston, 1985; Novikova et al., 2006). Systemic administration of MPTP (2 x 40 mg/kg, s.c.) to C57 BL/6 mice induced L-Dopa reversible hypoactivity (Fredriksson et al., 1990; Sundström et al., 1990). Less rigorous dosage regimes, such as 2 x 20, or 2 x 25 or 2 x 30 mg/kg of MPTP were shown not to affect motility in the C67 Black mice although losses of DA concentrations upto 50-80% may be observed (Heikkila et al., 1989; Sonsalla and Heikkila, 1986). The notion of the MPTP model of PD as an inflammatory condition following following toxic neurodegeneration has been suggested (Beal, 2003; Członkowska et al., 2000; Kurkowska-Jastrzebska et al., 1999). It has been shown that CNS inflammation may induce an increased risk factor for drug-induced CNS toxicity or chemically mediated PD whereby the prolonged toxicity of MPP(+) may be due to a decrease in brain cytochrome P450 metabolism that occurs during inflammation (Goralski and Renton, 2004) which may persist over several years (McGeer and McGeer, 2004). The observation of increased levels of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 concomitant with decreased levels of neurotrophins, such as brain-derived neurotrophic factor (BDNF) in the nigrostriatal region of postmortem brains and/or in the ventricular or lumbar cerebrospinal fluid (CSF) is associated patients with sporadic PD, MPTP-treated mice and 6-hydroxydopamine (6-OHDA)-induced PD in rats (Hald et al., 2007; Lofrumento et al., 2011; Miller et al., 2009; Nagatsu and Sawada, 2005). A chronic MPTP treatment regime elevated the pro-inflammatory cytokines, IL-1β, TFN-α, interferon (IFN)-gamma, granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-10 accompanied by striatal DA depletion, increased striatal DA and serotonin turnover and impaired rotarod performance (Luchtman et al., 2009). An essential aspect of the MPTP-induced inflammation is associated with microglial activation (Alvarez-Erviti et al., 2011; Chung et al., 2011; Soreq et al., 2012). In this regard, it has been found that wheel-running decreased the proportion of new microglia that were increased in aged mice thereby emphasizing the influence of exercise upon

microglial populations (Barrientos et al., 2011; Eyre and Baune, 2012; Kohman et al., 2012). The issue of exercise influencing neuroimmune functioning has been addressed; nevertheless, it remains a possibility that any interventions that may reinforce the effects of physical exercise may potentially lead to improvements in anti-inflammatory effects.

The major purpose of the present study was to was to examine whether or not the prepared yeast product, Milmed, may reinforce the ameliorative effects of physical exercise upon the MPTP-induced deficits, expressed in motor function and striatal DA, through twice weekly administration over seven weeks (cf. Archer and Fredriksson, 2013a). The ancilliary purpose was to examine whether or not the prepared yeast product, Milmed, may reinforce the ameliorative effects of physical exercise upon the MPTP-induced deficits through twice weekly administration over ten weeks.

Methods and materials

Animals. Male C57 Bl/6 mice were purchased from B&K, Sollentuna, Sweden, and were maintained, five-to-a-cage, in plastic cages in a room at temperature of 22 ± 1^{o}C and a 12/12 hours constant light/dark cycle (lights on between 06.00 and 18.00 hrs). They were placed and maintained in groups of 4 to 6 animals in a room maintained for male mice only following arrival at the laboratory for about 2 weeks in order to acclimatize. Free access to food and water was maintained throughout, except for the day previous to the initiation to wheel-running exercise which occurred at the end of the second week following arrival. They were housed in groups of 6 animals, wheel-running exercised and activity chamber tested only during the hours of light (08.00-15.00 hrs). All exercising and testing was performed in a normally lighted room. Half of the mice in each treatment condition (MPTP-Exer; MPTP-Exer-Milmed; and Vehicle) were given wheel-running exercise whereas the other half were placed in a clean laboratory cage for the same period in a room in which the running wheels were placed. Motor activity was tested in a specially arranged test room. This test room, in which all 12 ADEA activity test chambers, each identical to the home cage, were placed, was well-secluded and used only for this purpose. Each test chamber (i.e. motor activity test cage) was placed in a sound-proofed wooden box with 12 cm thick walls and front panels and a small double-glass window to allow observation; each box had a dimmed lighting.

Three weeks following arrival, four groups (n = 10) of DSP4-treated and two groups of vehicle-treated mice were administered either MPTP (2 x 40 mg/kg, s.c., 24 hours between injections) or vehicle (0.9% physiological saline injected s.c. in a volume of 2 ml/kg body weight). Milmed (see below for details of preparation) or vehicle were administered twice weekly.

Experiments were carried out in accordance with the European Communities Council Directive of 24th November 1986 (86/609/EEC) after approval from the local ethical committee (Uppsala University and Agricultural Research Council), and by the Swedish Committee for Ethical Experiments on Laboratory Animals (license S93/92 and S77/94, Stockholm, Sweden).

Drugs. MPTP (Research Biomedical Inc., MA, USA, 2 x 20 mg/kg or 2 x 40 mg/kg, s.c., with a 24-hr interval between injections in each case) was dissolved in saline and administered s.c. in a volume of 2 ml/kg body weight. Milmed yeast preparation was obtained through treatment and preparation of *Saccharomyces cerevisiae* with electromagnetic waves in the Extreme High Frequency (EHF) range of 30 GHz to 300 GHz to produce the treated yeast extract (cf. Golant, 1994). Saline was used as vehicle treatment in each case. Mice were orally treated with 0.5 ml Milmed containing approximately one million yeast cells daily according to the preparation protocol and design developed from previous observations regarding stability and viability of compound.

Milmed Preparation and administration

Milmed was obtained through treatment and preparation of the yeast fungi, *S. cerevisiae*, a strain that is obtained from the International Research Center ‘‘Beer and Beverage XXI Century’’, Moscow, Russia. The yeast was cultured in wort which was produced through meaded malt extract. The treatment of the yeast in a electromagnetic field of superhigh frequencies with electromagnetic waves in the EHF range of 30–300 GHz to produce the treated yeast extract (cf. Golant 1994), after which the yeast was re-cultured at 25–28^{o}C for 48 h. Saline was used as vehicle in each case. Following this, the cell concentration in the completed yeast suspension was measured using NucleoCounter YC-100 (ChemoMetec A/S, Denmark) and the extent to which the suspension should be diluted was decided. The yeast suspension was sent to the Department of Neuroscience

Laboratory at the University of Uppsala from Production Unit at Milmed AB Company (Färjestad, Sweden). A bottle with sterilised wort for dilution was sent concurrently. Mice were orally treated with 0.5 ml/kg Milmed containing a cell concentration of approximately 2 x 106 yeast cells daily according to the preparation protocol and design developed from previous observations regarding stability and viability of compound (each dose contained 1 x 10^6 yeast cells). Each mouse was administered Milmed once per day 4 times/week, per orally (p.o.), during the 10 (or 11 for the MPTP + Exer + M(ii) group): The administered suspension was prepared twice weekly and sent to the Uppsala University on Mondays and Tuesdays. The suspension delivered on Mondays was administered to the mice in the respective groups on Mondays and Tuesdays whereas that suspension delivered on Tuesdays was administered on Wednesdays and Thursdays (cf. Archer and Fredriksson, 2013a). Throughout, the suspension delivered to the laboratory was maintained at 5oC in the refrigerator.

Design and Treatment

Table 1. Chronological and procedural description of exercise schedules and Milmed treatment for MPTP-treated and control mice.

Week/Day	**Vehicle**	**MPTP**	**MPTP+Exer**	**MPTP+Exer+M(i)**	**MPTP+Exer+M(ii)**
Monday	cage	Cage	Cage	Cage	Exer+M
	cage	Cage	Cage	Cage	Exer+M
Week 1	cage	Cage	Cage	Cage	Exer+M
	cage	Cage	Cage	Cage	Exer+M
Friday	Test+sal	Test+MPTP	Test+MPTP	Test+MPTP	Test+MPTP
Monday	Exer	Cage	Exer	Exer+M	Exer+M
	Exer	Cage	Exer	Exer+M	Exer+M
Week 2-3	Exer	Cage	Exer	Exer+M	Exer+M
	Exer	Cage	Exer	Exer+M	Exer+M

Friday	Test+Sal	Test+MPTP	Test+MPTP	Test+MPTP	Test+MPTP
Monday	Exer	Cage	Exer	Exer+M	Exer+M
	Exer	Cage	Exer	Exer+M	Exer+M
Week 4-7	Exer	Cage	Exer	Exer+M	Exer+M
	Exer	Cage	Exer	Exer+M	Exer+M
Friday	Test	Test	Test	Test	Test
	Test	Test	Test	Test	Test
Week 8	Sacrifice and dissection of striatal regions				

M = Milmed; MPTP; Exer = exercise; Cage = placement in cages instead of exercise

On Wednesdays all the groups, including Vehicle and MPTP were placed in the running wheels for 30 min.

On Fridays, before testing in motor activity test cages, all the groups, including Vehicle and MPTP were placed in the running wheels for 10 min.

Behavioural Measurements and Apparatus. Activity test chambers: An automated device, consisting of macrolon rodent test cages (40 x 25 x 15 cm) each placed within two series of infra-red beams (at two different heights, one low and one high, 2 and 8 cm, respectively, above the surface of the sawdust, 1 cm deep), was used to measure spontaneous motor activity (RAT-O-MATIC, ADEA Elektronic AB, Uppsala, Sweden). The distance between the infra-red beams was as follows: the low levels beams were 73 mm apart lengthwise and 58 mm apart breadthwise in relation to the test chamber; the high level beams, placed only along each long side of the test chamber were 28 mm apart. According to the procedures described previously (Archer et al., 1986), the following parameters were measured: LOCOMOTION was measured by the low grid of infra-red beams. Counts were registered only when the mouse in the horizontal plane, ambulating around the test-cage. REARING was registered throughout the time when at least one high level beam was interrupted, i.e. the number of counts registered was proportional to the amount of time spent rearing. TOTAL ACTIVITY

was measured by a sensor (a pick-up similar to a gramophone needle, mounted on a lever with a counterweight) with which the test cage was constantly in contact. The sensor registered all types of vibration received from the test cage, such as those produced both by locomotion and rearing as well as shaking, tremors, scratching and grooming. All three behavioural parameters were measured over three consecutive 20-min. periods. The motor activity test room, in which all 12 ADEA activity test chambers, each identical to the home cage, were placed, was well-secluded and used only for this purpose. Each test chamber (i.e. activity cage) was placed in a sound-proofed wooden box with 12 cm thick walls and front panels, and day-lighting. Motor activity parameters were tested on one occasion only, over three consecutive 20-min periods, at the age of three to four months. Groups of mice were treated with MPTP and then given access to running wheels (30-min/day, 4 times/week), with or without concomitant treatment with Milmed ([Milm(1)-charged] or [Milm(0)]-uncharged=yeast itself), as displayed in Table 1.

Computer-linked running-wheel units

Small rodent exercise wheels for treadmill-type running behaviour, purchased from a Pet store and considered suitable for the C57/Bl6 mice to run upon. The wheels were adapted and modified for use by laboratory mice; they were placed altogether in a large sound-proofed room within the animal housing section of the laboratory. All 25 running wheels were placed equidistant from each other with adjacent wheels in two long rows such that the sounds of the 'wheel-revolving noises' emitted by any single wheel could be heard easily by the occupants of all the other 24 wheels. A photograph of the types of running wheels utilized in all our physical exercise studies, presenting a double row of the activity-enhancing running wheels applied in all the experiments as well as the "holding" cages in which the non-exercised groups remained, was depicted previously (Archer and Fredriksson, 2010). In previous neuroteratological studies that observed wheel-running exercise following different types of perinatal treatments, it was observed that each wheel needed to be placed in isolation from each of the others because the noise emitted from the wheel-running of any one animal served to evoke wheel-running behavior by the other animals. In the study by Archer and Fredriksson (2012, Experiment II), one group "MPTP+Wheel-NoEx" was placed in the running wheels for the 30-min exercise periods but in this case the wheels had been fixed and remained immobile despite efforts by each mouse to get the wheel to revolve. Thus, each mouse would initially attempt to run up the slope of the wheel but would soon give up and remain unexercised. It was observed that placement in the 'non-revolving' wheel result in

similar MPTP-induced deficits in function and striatal DA concentration. The Rodent running-wheels with computer-controlled devices adapted for measurement of running exercise per unit time (Ödman et al., 2013; Archer and Fredriksson, 2013a). The arm of the upright (1) registers every revolution which is fed into a laptop computer that monitors all running activity by each mouse during the wheel-running session (see Figure 1), The number of revolution per unit time is converted automatically to "distance run" per unit time and mouse, and thereafter available for statistical analysis.

Insert Figure 1 here

Procedure. In present experiment, mice in the non-exercised, sedentary groups were placed in the holding cages singly for 3 out of the 4 days (Mondays, Tuesdays and Thursdays) that the exercised mice were given access to the running wheels. On Wednesdays, all the mice, in both the exercised and non-exercised groups were given 30-min access sections in the running-wheels: Thus, the exercise condition is defined by four 30-min sessions/week in the running wheels whereas the non-exercise condition is defined by a single 30-min session/week in the running wheels. The single 30-min session (Wednesdays) was taken as an exercise test session in order to monitor the extent of exercise by each group. Each of the running wheels was monitored by a laptop computer that registered every revolution (utilizing arm responding to each wheel revolution) and counted revolutions per unit time during each session (see Figure 1). On Fridays, prior to the tests of spontaneous motor activity in the motor activity test chambers (60-min test sessions), a single 10-min test session/week was given all the mice in the exercise and non-exercise conditions. Following the 60-min spontaneous motor activity test during Week 10 (Friday), each mouse was injected a subthreshold dose of L-Dopa (5 mg/kg, s.c.) and replaced in its motor activity test chamber and locomotor, rearing and total activity counts were registered over a further 180 min. During Week 11, all the mice were sacrificed and striatal regions taken for analysis of DA concentrations. Each of mice in each of the five different was identified through careful marking procedures so that the DA concentration, running distances from the 30-min (Wednesday) and 10-min (Friday) tests during Weeks 1 to 10, locomotor, rearing and total activity counts from the tests of spontaneous motor activity tests during Weeks 1 to 10, locomotor, rearing and total activity counts from the L-Dopa-induced motor activity test on Week 10 only and DA concentrations of each mouse were registered and identified.

Neurochemical analysis. Mice were killed by cervical dislocation within two weeks of completion of behavioural testing. Determination of DA was performed using an high-performance liquid chromatograph with electrochemical detection (HPLC-EC), according to (Björk et al. 1991), as modified by (Liu et al. 1995). Striatal regions were rapidly dissected out and stored at -80(C until neurochemical analysis. DA concentration was measured as follows: The frozen tissue samples were weighed and homogenized in 1 ml of 0.1 M perchloric acid, and alpha-methyl-5-hydroxytryptophan was added as an internal standard. After centrifugation (12 000 rpm, i.e. 18600 g, 4oC, 10 min) and filtration, 20 µl of the supernatant was injected into the HPLC-EC to assay DA. The HPLC system consisted of a PM-48 pump (Bioanalytical Systems, BAS) with a CMA/240 autoinjector (injection volume: 20 µl), a precolumn (15 x 3.2 mm, RP-18 Newguard, 7 µm), a column (100 x 4.6 mm, SPHERI-5, RP-18, 5 µm), and an amperometric detector (LC-4B, BAS, equipped with an Ag/AgCl reference electrode and a MF-2000 cell) operating at a potential of +0.85V. The mobile phase, ph 2.69, consisted of K2HPO4 and citric acid buffer (pH 2.5), 10% methanol, sodium octyl sulphate, 40 mg/l, and EDTA. The flow rate was 1 ml/min, and the temperature of the mobile phase was 35^{o}C.

Statistical analysis. The distance run over 30-min and 10-min sessions, as well as the number of rotations in the running wheels, and the locomotion, rearing and total activity counts during the tests of spontaneous motor activity over 7 weeks of testing were analysed using Split-plot ANOVA. L-Dopa-induced activity during the final, week-7 test and DA concentrations in the striatum were analysed using one-way ANOVA. Pairwise comparisons using Tukey's HSD test were performed.

Results

All three MPTP groups that received exercise (4 sessions/week) increased the number of rotations and the mean distance run from week 2 to week 7 whereas neither the vehicle nor the MPTP (exercise only on Wednesdays) groups did so. Split-plot ANOVA indicated significant Treatment x Test week interaction effects for Number of rotations: $F(34, 419) = 119.34$, $p < 0.0001$; and Distance run: $F(34, 419) = 76.63$, $p < 0{,}001$. Figure 2 presents

Insert Figure 2

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Insert Figure 3 here

All three MPTP groups that received exercise (4 sessions/week) increased all three parameters of spontaneous motor activity, locomotion, rearing and total activity, from week 2 to week7. Split-plot ANOVA indicated Treatment x Test week interaction effect: $F(34, 419) > 54.71$, $p < 0.0001$, for locomotion, rearing and total activity. Figure 4 presents the mean Locomotion, Rearing and Total activity over 60-min in the motor activity test boxes (on Fridays) by MPTP mice given four-day/week access (MPTP+Exercise) or only one day/week access (Wednesdays, MPTP), and either Milmed (Yeast) treatment or not, and vehicle-treated mice.

Insert Figure 4 here

Tukey-HSD tests indicated the following differences:-
Locomotion:
Week 2, 3 and 5: MPTP < MPTP+Exer, MPTP+Exer+MilmedI, MPTP+Exer+MilmedII < vehicle
Week 4, 6 and 7: MPTP < MPTP+Exer, MPTP+Exer+MilmedI, MPTP+Exer+MilmedII < vehicle

Rearing:
Week 2: MPTP, MPTP+Exer < MPTP+Exer+MilmedI, MPTP+Exer+MilmedII < vehicle
Week 3 and 4: MPTP < MPTP+Exer, MPTP+Exer+MilmedI, MPTP+Exer+MilmedII < vehicle
Week 5, 6 and 7: MPTP < MPTP+Exer, MPTP+Exer+MilmedI, MPTP+Exer+MilmedII, vehicle

Total activity:
Week 2: MPTP, MPTP+Exer < MPTP+Exer+MilmedI, MPTP+Exer+MilmedII < vehicle
Week 3, 4 and 5: MPTP < MPTP+Exer, MPTP+Exer+MilmedI, MPTP+Exer+MilmedII < vehicle
Week 6 and 7: MPTP < MPTP+Exer, MPTP+Exer+MilmedI, MPTP+Exer+MilmedII, vehicle

The locomotor paramater of L-Dopa-induced activity was increased by the wheel-running physical exercise schedule, although in the case of the Milmed groups (MPTP+Exer+MilmedI and MPTP+Exer+MilmedII groups) L-Dopa-induced activity was restored completely by Test week 7 for all three parameters of activity. One-way ANOVA indicated a significant Treatment effect: $F(4, 34) = 35.02$, $p < 0.0001$. Figure 5 presents

Insert Figure 5 here

Tukey-HSD tests indicated the following differences:-
Locomotion: MPTP < MPTP+Exer < MPTP+Exer+MilmedI, MPTP+Exer+MilmedII, vehicle
Rearing and Total activity: MPTP, MPTP+Exer, MPTP+Exer+MilmedI, MPTP+Exer+MilmedII, vehicle.

The physical exercise schedule attenuated the DA deficit of the MPTP-treated mice (MPTP vs MPTP+Exercise). However, the combination of Milmed with wheel-running exercise restored DA concentrations in MPTP mice completely. One-way ANOVA indicated a significant Treatment effect $F(4, 25) = 67.40$, $p < 0.0001$. Figure 6 presents Dopamine concentrations of groups of mice administered MPTP (3 x 30 mg/kg) and vehicle with either four-day/week access (MPTP+Exercise) or only one day/week access (Wednesdays, MPTP), and either Milmed (Yeast) treatment or not.

Insert Figure 6 here

Tukey-HSD test indicated the following differences: MPTP < MPTP+Exer < MPTP+Exer+MilmedI, MPTP+Exer+MilmedII, vehicle.
Percent of control (vehicle) values were:

MPTP (25%),MPTP+Exer (47%),MPTP+Exer+MilmedI (89%),MPTP+Exer+MilmedII (106%).

Discussion

The present findings indicate that the combination of physical exercise (wheel-running) with four times (with two batches of the yeast delivered each week) weekly administrations of Milmed abolished the MPTP-induced functional and DA deficits and restored both the length of distance run during each wheel-running session, locomotor, rearing and total activity during testing each week, L-Dopa-induced activity and DA concentration in the striatum. Over the course of six weeks (from Week 2 to Week 7), the MPTP-induced deficits in the length of distance run during each wheel-running session, locomotor, rearing and total activity during testing each week, L-Dopa-induced locomotor activity and DA concentration in the striatum were all alleviated by the wheel-running exercise regime alone. Nevertheless, the combination of exercise with the product, Milmed, was necessary to abolish the functional motor and neurochemical deficits. The above descriptions of animal models of Parkinsonism presented the necessity of 'fusion' models that combine neurotoxic actions at mitochondrial sites with genetic predispositions for neuronal loss. The experimental results presented above refer to the particular characteristic of DA neurons: "use it or lose it". In several studies (Archer and Fredriksson, 2010, 2012, 2013a, b), employing several different dosage regimes of MPTP, we have shown that wheel-running as physical exercise regularly alleviates the functional deficit and to a lesser extent, the DA deficit as a function of the length of recovery time (upto 14 weeks) that was allowed. However, previously, restoration of both functional and DA deficits was never found to be complete (Archer and Fredriksson, 2010, 2012; Fredriksson et al., 2011). In several studies, it has now been observed that the fusioning of physical exercise with the 'treated yeast', Milmed, both restores the functional deficits and the DA deficits caused by MPTP (Archer and Fredriksson, 2013a).

The loss of DA following MPTP administration varies quite systematically according to dose levels and number of administrations: for example, following MPTP (2 x 40 mg/kg, 24 hour interval between injections) the level of DA was 17% of control values in agreement with large number of previous studies (e.g. Archer and Fredriksson, 2010); following MPTP (4 x 40 mg/kg, one week intervals between injections) it was 11% to 17% of

controls; following MPTP (4 x 30 mg/kg, one week intervals between injections) it was 19% to 20% controls; finally, applying the present MPTP dose regime of 2 x 30 mg/kg, separated by a one-week interval, it was 25% of control values. It must be considered that in the last case only the MPTP (no exercise) received exposure to the running-wheel during a 30-min once a week only (Wednesdays). In a recent experiment, as yet unpublished, the DA level of MPTP receiving the single exercise session was 24% of control values; it appears that with the 2 x 30 mg/kg, single exercise session, regime 24-25% of controls may be consistent. Gerecke et al. (2010) employed a MPTP regime of 4 x 20 mg/kg administered at 2-hr intervals and either allowed an unrestricted wheel-running exercise schedule or a partial exercise schedule or no exercise activity during a 90-day period. Approximate estimations of the DA concentrations in the substantia nigra pars compacta region, through extrapolation of their data presented in Figure 3 (Gerecke et al., 2010), suggest that DA levels in the MPTP (unexercised group, termed SH + MPTP) were 38% of the control group values (termed SH +SH) whereas their MPTP-exercise group (termed 3M EX + MPTP) indicated 60% of control values. For the MPTP+exercise group (four 30-min sessions/week) in the present study, the level of DA was 47% of control values after six weeks' access to the running-wheels. In the recent unpublished experiment (referred to above), all the conditions were identical except that nine-weeks' of exercise intervention (rather than six) was provided and), the level of DA was 51% of control values; once again, it appears that restorative effects of exercise alone are limited. In the Archer and Fredriksson (2012) study, employing a 4 x 30 mg/kg MPTP regime in both experiments, DA concentrations of the unexercised MPTP groups were 20% and 19% (exercise intervention was introduced much too late) and 23% and 20%, respectively, of control values while that of the MPTP-Exercise groups was 39% and 41%, respectively (Experiments I and II), of control values. Taken together with the results of the Fredriksson et al. (2011) results, these above indications and several other studies employing exercise intervention against MPTP deficits show that exercise alone, despite its utility in Parkinsonism and other neurodegenerative/neuroinflammatory conditions (Archer, 2011, 2012; Archer and Kostrzewa, 2012; Archer et al., 2011a, b; Archer et al., 2012), only provides a partial restoration. Nevertheless, the role of exercise appears central: as Tuon et al. (2012) imply, it may be that the effects of exercise on PD deficits may be, at least partially, to modulate the neurochemical status in the striatum of rats, possibly by improving the oxidative stress parameters.

It is becoming increasing clear that the combination of exercise intervention with the treated yeast, Milmed, administration two to four times during each week of the wheel-running schedule regularly provides a complete restoration of function and DA deficits (cf. Archer and Fredriksson, 2013a; unpublished data). In the case of the MPTP+Exer+Milmed** group, both running wheel exercise and the yeast treatment were initiated the same week as the 1st MPTP administration, whereas in the case of MPTP+Exer+Milmed* group, running wheel exercise and the yeast treatment were initiated the following week. The 1st injection of MPTP reduced running wheel activity, for both the 30-min (Wednesday) test and the 10-min (Friday) test, in both these groups, lesser so in the case of the former, but reduced all three parameters of spontaneous motor activity, locomotion, rearing and total activity, to a much greater extent: the reductions of wheel-running activity from the 1st to the 2nd week for the MPTP+Exer+Milmed** group was 13% for the 10-min test and 22% for the 30-min test whereas the reduction of spontaneous locomotor behaviour was 66%; the reductions of wheel-running activity from the 1st to the 2nd week for the MPTP+Exer+Milmed* group was 38% for the 10-min test and 39% for the 30-min test whereas the reduction of spontaneous locomotor behaviour was 73%. All these percentage reductions refer to activity comparisons between the 1st and 2nd weeks. It ought to be noted too that extent of recovery as measured through running wheel performance or spontaneous motor activity differed also, but to a much lesser extent, for the MPTP+Exer+Milmed* group but not the MPTP+Exer+Milmed** group which recovered to a level exceeding that of the vehicle group. In this case it was observed that increase in running distance from the 2nd to the 7th week was 72% for the 30-min test and 71% for the 10-min test whereas the spontaneous locomotor activity increase was 66%. DA concentrations in the striatum were shown to be 89% of control values for the MPTP+Exer+MilmedI and 106% for the MPTP+Exer+MilmedII group, respectively. In neither case was there any significant difference compared to vehicle controls. The conclusion that whereas exercise by itself ameliorated function and DA integrty only partially the fusion of exercise with Milmed administration induced complete recovery is compelling.

It was observed previously that, in addition to abolishing the functional motor and neurochemical (DA) deficits, the exercise-Milmed co-administration induced also a profound elevation of brain-derived neurotrophic factor (BDNF) levels in the parietal region, that included the motor cortex, of the MPTP-treated mice (Archer and Fredriksson, 2013b). It is interesting to note that memantine, a medium-affinity, uncompetitive receptor antagonist of N-methyl-D-aspartate, that has been administered clinically as a neuroprotective agent to treat

Alzheimer's disease and PD, increased BDNF mRNA concentrations markedly in limbic cortex regions at clinically relevant doses (Marvanova et al., 2001).

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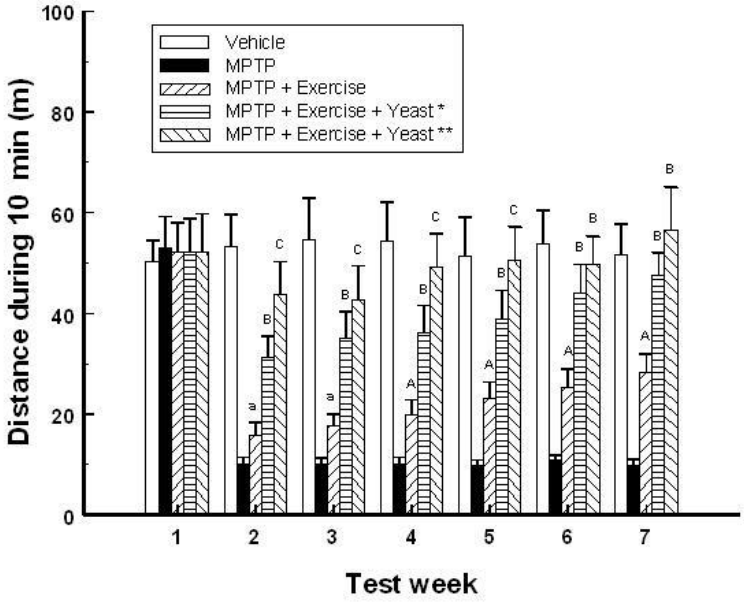
Figure 1. Computerized running wheels used for the 30-min or 10-min exercise sessions and for registration of number of revolutions/session from which was derived distance run.

Figure 2. Mean distance (SD) run (above) and mean number of rotations (SD) by MPTP mice given four-day/week access (MPTP+Exercise) or only one day/week access (Wednesdays, MPTP), and either Milmed (Yeast) treatment or not, and vehicle-treated mice, during the 10-min running test prior to testing of spontaneous motor activity in the ADEA test chambers.



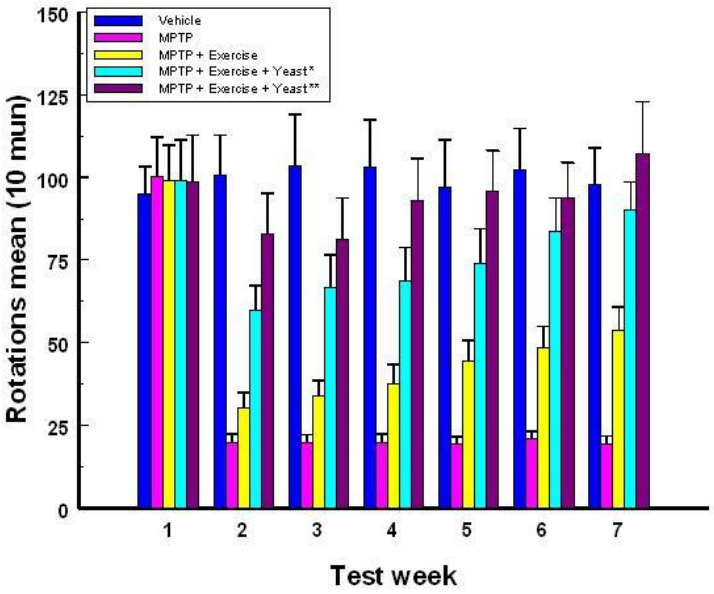


Figure 3. Mean distance (SD) run by MPTP mice given four-day/week access (MPTP+Exercise) or only one day/week access (Wednesdays, MPTP), and either Milmed (Yeast) treatment or not, and vehicle-treated mice, during the 30-min Wednesday tests.

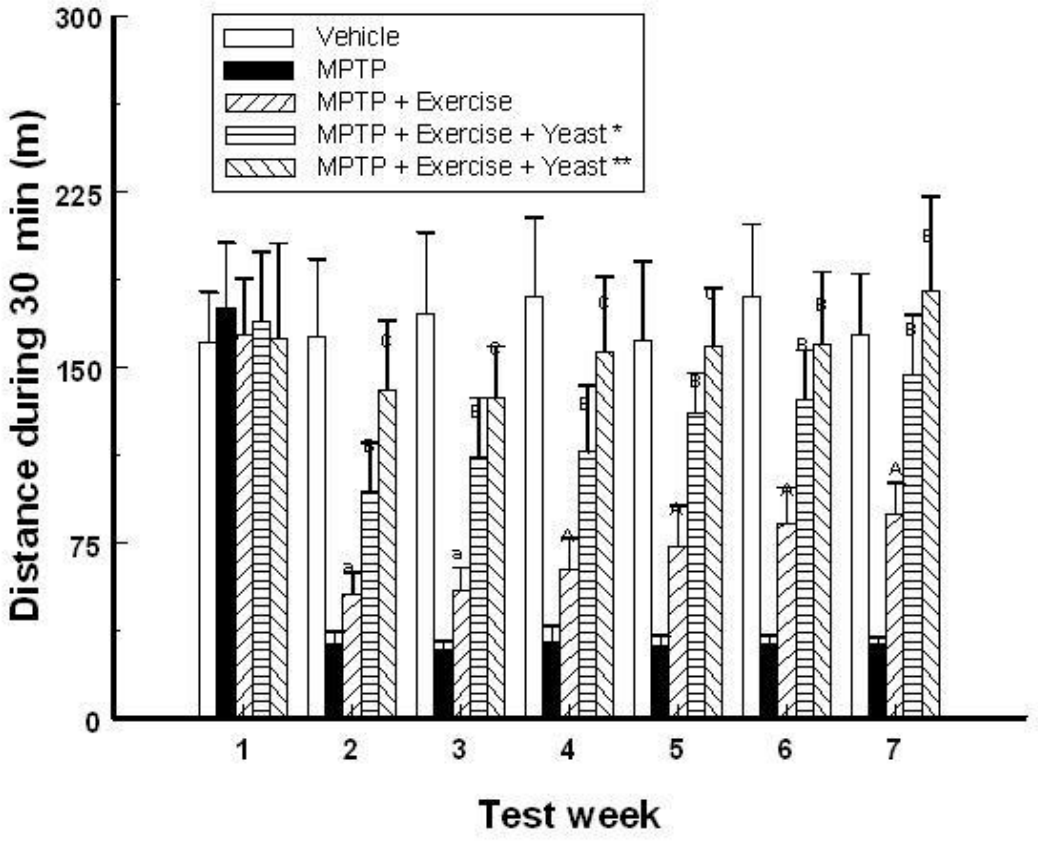


Figure 4. Mean Locomotion, Rearing and Total activity over 60-min in the motor activity test boxes (on Fridays) by MPTP mice given four-day/week access (MPTP+Exercise) or only one day/week access (Wednesdays, MPTP), and either Milmed (Yeast) treatment or not, and vehicle-treated mice.

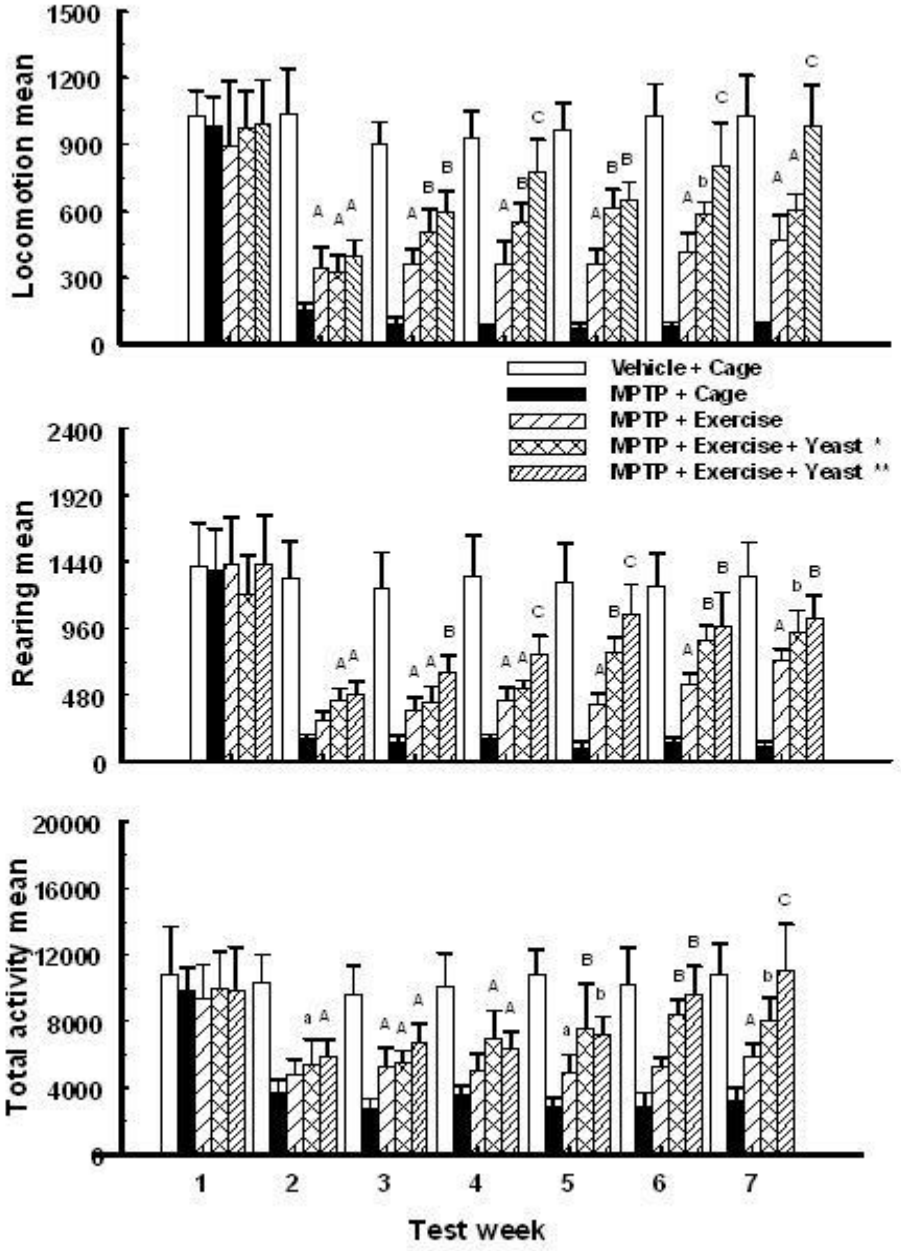
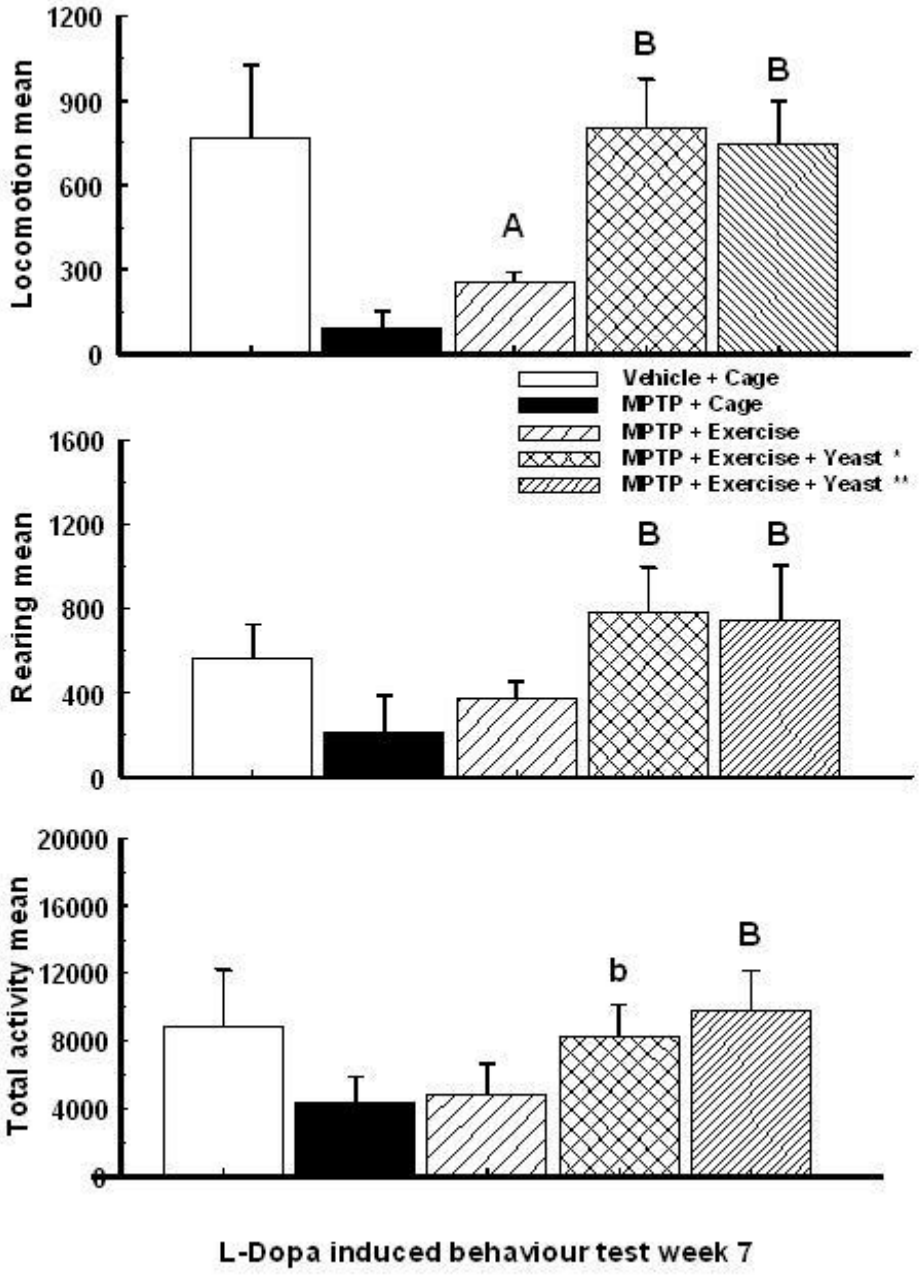


Figure 5. Mean (SD) locomotion, rearing and total activity counts for each of the five groups: Vehicle, MPTP, MPTP+Exercise, MPTP+Exercise+MilmedI (MPTP+Exercise+Yeast*) and MPTP+Exercise+MilmedII (MPTP+Exercise+Yeast**) over 180-min test sessions for L-Dopa-induced activity at testing day (Fridays) during Week 7 only. For locomotion, MPTP+cage < MPTP+Exercise; A versus MPTP+Exercise ($p < 0.01$), a $p < 0.05$.



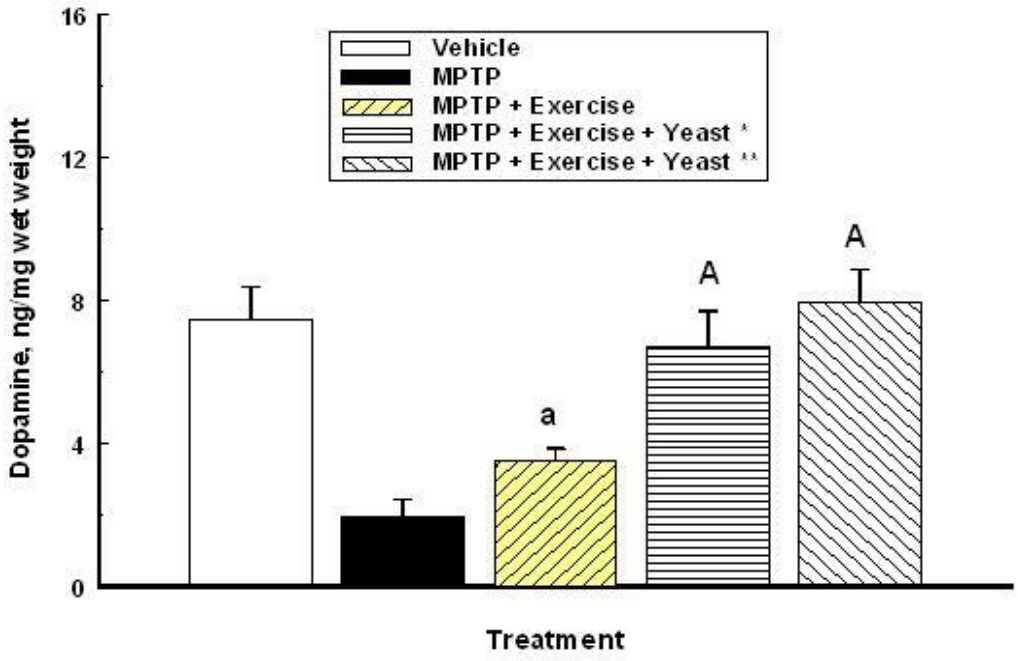


Figure 6. Mean (SD) concentrations of striatal dopamine for each of the five groups: Vehicle, MPTP, MPTP+Exercise, MPTP+Exercise+MilmedI (MPTP+Exercise+Yeast*) and MPTP+Exercise+MilmedII (MPTP+Exercise+Yeast**). A versus MPTP; B versus MPTP+Exercise.