Effect of MPTP on mRNA expression of PGC-1α in mouse brain

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Abstract

The peroxisome proliferator-activated receptor-γ (PPARγ) coactivator 1α (PGC-1α) is a key regulator of mitochondrial biogenesis, respiration and adaptive thermogenesis. Beside the full-length protein (FL-PGC-1α), several other functionally active PGC-1α isoforms were identified as a result of alternative splicing (e.g., N-truncated PGC-1α; NT-PGC-1α) or alternative promoter usage (e.g., central nervous system-specific PGC-1α isoforms; CNS-PGC-1α). The achievement of neuroprotection via the CNS-targeted pharmacological stimulation is limited due to the poor penetration of the blood brain barrier (BBB) by the proposed pharmaceutical agents, so preconditioning emerged as another option. The current study aimed at the examination of how the expression levels of FL-, NT-, CNS- and reference PGC-1α isoforms change in different brain regions following various 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment regimens, including the chronic low dose treatment for preconditioning. Ninety minutes following the acute treatment regimen, the expression level of FL-, NT- and CNS-PGC-1α isoforms increased significantly in the striatum, cortex and cerebellum. However, this elevation was diminished 7 days following the last MPTP injection in this acute treatment regimen. The chronic low dose administration of MPTP, which did not cause significant toxic effect in light of the relatively unaltered dopamine levels, neither resulted in any significant change of PGC-1α expression as well. The elevation of PGC-1α levels following acute treatment may demonstrate a short-term compensatory mechanism against the mitochondrial damage induced by the complex I inhibitor MPTP. However, drug-induced preconditioning by chronic low dose MPTP seems not to induce protective responses via the PGC-1α system.

Keywords: FL-PGC-1α, NT-PGC-1α, CNS-PGC-1α**,** MPTP, preconditioning, dopamine.

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by the loss of dopaminergic neurons, and the presence of Lewy bodies in the substantia nigra (SN) pars compacta (Forno, 1996). Although the precise pathomechanism of PD is not fully understood, several molecular mechanisms of neuronal death were described in the pathogenesis, including mitochondrial dysfunction, energy deficit and oxidative stress (Bose and Beal, 2016). It is postulated that the life-long cumulative low dose exposition to mitochondrial toxins may contribute to the pathogenesis of certain neurodegenerative disorders (Harris and Blain, 2004). The delineation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced Parkinsonian symptoms yielded one of the first evidence that mitochondrial dysfunction is involved in PD pathogenesis (Forno et al., 1993). Accordingly, systemic MPTP administration have been widely used to study disease mechanisms in various in vivo animal studies (Javitch et al., 1985).

Beside environmental factors, several causative or susceptibility genes have been identified in PD, many of them having direct implications in mitochondrial dysfunction (Kalinderi et al., 2016). Peroxisome proliferator-activated receptor-gamma (PPARγ) coactivator-1 alpha (PGC-1α) is one of them, which may play a role in PD pathogenesis as well. PGC-1α is a multifunctional transcriptional coactivator of nuclear respiratory factor 1 and 2 (NRF-1, -2), estrogen-related receptors (ERRs) and PPARs amongst others, and hereby regulates mitochondrial function and biogenesis (Knutti and Kralli, 2001).

Analysis of human brain samples indicated that PD is associated with the increased methylation of PGC-1α promoter and the reduced expression of PGC-1α itself (Su et al., 2015), and its downstream-regulated genes in the SN of PD patients (Zheng et al., 2010). Furthermore, possible associations of PGC-1α polymorphisms with PD risk, age of onset and longevity were described as well (Clark et al., 2011). Reduced expression of PGC-1α leads to enhanced α-synuclein oligomerization, too (Ebrahim et al., 2010), and accordingly,

overexpression of PGC-1α produced neuroprotection against α-synuclein- and rotenone-induced neurotoxicity (Zheng et al., 2010).

Several PGC-1α isoforms were identified as a result of alternative splicing and alternative promoter usage (Martinez-Redondo et al., 2015). The proximal promoter of PGC-1α has been reported as an important key regulator in several neurodegenerative diseases, including PD (Su et al., 2015). With regard to alternative splicing, beside the full-length protein (FL-PGC-1α), the N-truncated PGC-1α (NT-PGC-1α) isoform was discovered next, which is a shorter, but active isoform of PGC-1α (Zhang et al., 2009). Recent studies identified further different tissue-specific isoforms of PGC-1α, including central nervous system-specific isoforms (CNS-PGC-1α (Ruas et al., 2012; Soyal et al., 2012)). The novel CNS-specific isoforms originated from a new promoter located 587 kb upstream of exon 2 (Choi et al., 2013; Soyal et al., 2012). A recent study demonstrated that both PGC-1α reference gene and CNS-PGC-1α are downregulated in human PD brain and in experimental models with α-synuclein oligomerization and that the pharmacological activation or genetic overexpression of PGC-1α reference gene reduced α-synuclein oligomerization and toxicity (Eschbach et al., 2015). In contrast, the loss of that PGC-1α enhances the vulnerability of SN pars compacta dopaminergic neurons to α-synuclein toxicity (Ciron et al., 2015). These data suggest that PGC-1α downregulation and α-synuclein oligomerization form a vicious circle (Eschbach et al., 2015). Similarly to PD, certain mutations in amyotrophic lateral sclerosis inhibit the expression of CNS-specific isoforms, indicating this as a common finding in neurodegeneration (Bayer et al., 2017).

St-Pierre et al. described that PGC-1α-deficient mice are more sensitive to MPTP toxicity compared to the controls (St-Pierre et al., 2006). Interestingly, the sub-chronic administration of MPTP to mice resulted in the significant elevation of PGC-1α expression in the striatum after 24 hours that was normalized following 72 hours (Swanson et al., 2013). This may

represent an adaptive mechanism to neurotoxicity. Accordingly, the protective effect of PGC-1α was demonstrated previously as well; pioglitazone- and resveratrol-induced activation of PGC-1α was protective against MPTP toxicity (Breidert et al., 2002; Dehmer et al., 2004). However, there is a seeming controversy with regard to the effect of genetically induced overexpression of PGC-1α on MPTP neurotoxicity. On the one hand, the transgenic overexpression of PGC-1α was proved to protective against MPTP (Mudo et al., 2012), on the other hand, the adenovirus vector-mediated overexpression of PGC-1α resulted in dopamine depletion in the SN (Ciron et al., 2012) and consequently enhanced susceptibility to MPTP (Clark et al., 2012). The clarification of this issue needs further studies.

Evidence suggests the beneficial role of the stimulation of PGC-1α in neurodegenerative disorders. However, the CNS-targeted pharmacological stimulation is limited due to the poor penetration of the blood brain barrier (BBB) of the above-mentioned compounds, so preconditioning emerged as another option to achieve neuroprotection. It was previously demonstrated in light of our previous results that the acute administration of the selective complex II inhibitor 3-nitropropionic acid (3-NP) increased the expression of both FL- and NT-PGC-1α isoforms in the striatum of C57Bl/6 mice (Torok et al., 2015). As the available data are considerably limited with regard to the alteration of tissue-specific PGC-1α expression in the brain following MPTP administration, this study aimed at the examination of the expression levels of several PGC-1α isoforms in different brain regions following various MPTP treatment regimens. The hypothesis that low doses of MPTP may produce compensatory, protective alterations in the PGC-1α system was tested as well.

2. Results

2.1 Gene expression analysis

Ninety minutes following the last MPTP injection of the acute treatment of MPTP, the FL-PGC-1α and NT-PGC-1α expression significantly increased in the striatum (FL-PGC-1α: ctrl: 0.97 (0.92-1.04), MPTP: 1.47 (1.21-1.83), p = 0.0048; NT-PGC-1α: ctrl: 0.44 (0.40-0.49), MPTP: 0.70 (0.56-0.78), p = 0.019), cortex (FL-PGC-1α: ctrl: 0.96 (0.91-1.06), MPTP: 1.23 (1.15-1.43), p = 0.009; NT-PGC-1α: ctrl: 0.46 (0.43-0.48), MPTP: 0.69 (0.59-0.71), p = 0.0012) and cerebellum (FL-PGC-1α: ctrl: 1.50 (1.27-1.90), MPTP: 2.40 (2.07-2.76), p = 0.013; NT-PGC-1α: ctrl: 0.67 (0.48-0.86), MPTP: 1.21 (1.14-1.44), p = 0.009) (Fig. 1A, B). Furthermore, the MPTP-induced increases in the CNS-PGC-1α expression were also significantly larger in all investigated brain regions compared to the controls (striatum: ctrl: 1.03 (0.88-1.11), MPTP: 1.38 (1.34-1.78), p = 0.0069; cortex: ctrl: 0.91 (0.80-0.98), MPTP: 1.41 (1.24-1.42), p = 0.0048; cerebellum: ctrl: 1.51 (1.20-1.98), MPTP: 2.77 (2.34-3.17), p = 0.019) (Fig. 1C). However, there was not any difference between the Ref-PGC-1α levels in the striatum (ctrl: 0.11 (0.10-0.12), MPTP: 0.11 (0.95-0.12)), cortex (ctrl: 0.11 (0.11-0.12), MPTP: 0.09 (0.08-0.10)) and cerebellum (ctrl: 0.21 (0.20-0.29), MPTP: 0.28 (0.24-0.29)) of MPTP-treated and control mice (Fig. 1D).

One week following the last injection in the acute treatment regimen, there was not any significant change either in the FL-, NT-, CNS-, or in the Ref-PGC-1α levels between the control and the MPTP-treated animals in neither brain area (Fig. 2A-D).

Furthermore, the low-dose 12-day MPTP-treatment did not influence the expression levels of FL-PGC-1α, NT-PGC-1α, CNS-PGC-1α and Ref-PGC-1α as well in neither brain region (Fig. 3.A-D).

2.2 HPLC measurement

The dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) values in the respective control groups of the 3 treatment regimens were compared to each

other, and there were no significant differences. Therefore the values in these control groups were pooled for further comparisons with the MPTP-treated groups. The MPTP administration caused significant reductions in the striatal DA (ctrl: 8.08 ± 0.50, MPTP: 4.36 ± 0.92, p = 0.0005), DOPAC (ctrl: 2.57 ± 0.21, MPTP: 0.44 ± 0.08, p = $3.78*10^{-8}$) and HVA (ctrl: 2.18 ± 0.12, MPTP 0.67 ± 0.11, p = $5.12*10^{-10}$) levels compared to control values 90 min following its last administration in the acute treatment regimen (acute-1 day; Fig. 4). Moreover, a significant reduction in metabolite levels was also observed one week after the last injection in the acute treatment regimen (acute-7 days; Fig. 4) in the DA (ctrl: 8.08 ± 0.50, MPTP: 1.34 ± 0.43, p = $4.86*10^{-8}$), DOPAC (ctrl: 2.57 ± 0.21, MPTP: 0.76 ± 0.15, p = $7*10^{-6}$) and HVA (ctrl: 2.18 ± 0.12, MPTP: 0.81 ± 0.13, p = $5.08*10^{-8}$) values in the striatum of the MPTP-treated mice compared to the control animals. However, chronic MPTP treatment resulted in significant reductions of only striatal HVA (ctrl: 2.18 ± 0.12, MPTP 1.40 ± 0.08, p = 0.0005) levels, striatal DA (ctrl: 8.08 ± 0.50, MPTP: 6.83 ± 0.48) and DOPAC (ctrl: 2.57 ± 0.21; MPTP 1.99 ± 0.23) levels were not decreased significantly (Fig. 4). Seven days following the acute treatment regimen the DA levels significantly decreased compared to those data from samples obtained 90 min following the last MPTP injection in the acute treatment regimen (p = 0.039).

3. Discussion

PGC-1α is essential in normal mitochondrial functioning and its deficiency may contribute to neurodegeneration, while its stimulation demonstrated to be neuroprotective in certain models (Breidert et al., 2002; Dehmer et al., 2004; Eschbach et al., 2015; Mudo et al., 2012). Accordingly, the pharmacological induction of PGC-1α expression may come into account as a neuroprotective approach, but this possibility currently seems to be limited in light of the reduced BBB penetration of the potential pharmaceutical agents.

The aim of the current study was the thorough assessment of the expression of PGC-1α isoforms in various brain regions following different MPTP administration regimens, including a low-dose chronic one possibly mimicking drug-induced preconditioning.

Ninety minutes following the last MPTP injection of the high-dose acute treatment regimen of MPTP (75 mg/kg/day total dose) the expression level of FL-, NT- and CNS- PGC-1α isoforms increased significantly in the striatum, cortex and cerebellum. However, this elevation was diminished 7 days following the last MPTP injection in the acute treatment regimen. Torok et al. (Torok et al., 2015) demonstrated that the acute (90 min following a single dose injection of 100 mg/kg dose), but not the subacute (50 mg/kg twice daily for 5 days) 3-NP treatment regimen induced the overexpression of FL- and NT- PGC-1α isoforms mainly in the striatum (3-NP is a rather selective striatal neurotoxin (Brouillet et al., 2005)) similarly to the results of the current study. Those findings were explained by the probably reduced neuronal adaptive capability of the striatum following the neurotoxic insult. The above-mentioned results of the current study may also be explained by the further propagation of the neurotoxic process following 7 days in the acute treatment regimen in light of the extent of decreases in the striatal DA levels. The elevation of PGC-1α expression especially that of the CNS-specific isoform, may indicate a short-term compensatory protective mechanism against the mitochondrial dysfunction induced by the complex I inhibitor MPTP.

It is hard to interpret the increased expression of PGC-1α in the cerebellum, which is not primarily affected in MPTP toxicity. However, several lines of evidence indicated that MPTP neurotoxicity is not highly selective to dopaminergic neurons; in specific circumstances systemic MPTP administration resulted in Purkinje cell loss (Takada et al., 1993). The involvement of the cerebellum in disease mechanisms of different neurodegenerative disorders such as amyotrophic lateral sclerosis, Huntington’s disease (HD) and PD is frequently seen (Rees et al., 2014; Tan et al., 2016; Wu and Hallett, 2013). Furthermore,

increasing evidence suggest that PGC-1α expression is associated with degenerative changes in the CNS, including in the cerebellum (Torok et al., 2015). It was hypothesized that the elevation of PGC-1α in the cerebellum is a compensatory mechanism against the energy deficit which may be an important factor underlying the relative resistance of cerebellar neurons against neurodegenerative processes in HD and in PD.

The drug-induced preconditioning applying low-dose neurotoxic agents may stimulate neuroprotective mechanisms resulting in the amelioration of neurodegenerative process. This approach has already been demonstrated to be beneficial in case of 3-NP: the low-dose of toxin treatment increased the tolerance to ischemia and hypoxia in rats and gerbils (Horiguchi et al., 2003; Riepe et al., 1997; Wiegand et al., 1999). Although the exact mechanism in the background is not fully understood, the overexpression of free radical scavenging enzymes may be involved: acute 3-NP treatment activated superoxide dismutase (SOD) and catalase (CAT) in several brain areas (Binienda et al., 1998). Similarly, an increase in SOD activity in the glial cells of the striatum and SN was observed as well following MPTP treatment (Kurosaki et al., 2004). The preconditioning by MPTP is not intended to suggest future direct therapeutic approach, but rather aim at finding key players in the background which may help to do the best measures to alleviate the pathological alterations. The situation may be similar to ischemic preconditioning where the outcome in myocardial infarction may substantially depend on which medications were applied with an influence on preconditioning (Tomai et al., 1999). This may be especially important in light of the fact that environmental toxins could play a role in the pathogenies of idiopathic PD. The chronic low dose administration of MPTP in the current study neither resulted in significant DA depletion (i.e. neurotoxic effect at biochemical level), nor in any significant change of PGC-1α expression. These data suggest that drug-induced preconditioning by MPTP may not evoke apparent responses in the PGC-1α system.

In conclusion the current study demonstrated that acute severe mitochondrial dysfunction initiated protection via elevating the expression of brain specific isoforms of mitochondrial master regulator PGC-1α. However, low-dose chronic administration of mitochondrial toxin MPTP did not induce those protective mechanisms with the involvement of PGC-1α.

3. Experimental procedures

3.1 Animals

12-week-old C57Bl/6J male mice were used in this study. The animal strain was originally obtained from Jackson Labs (Jackson Laboratories, Bar Harbor, ME, USA).

The animals were housed in cages and maintained under standard laboratory conditions with 12-12 h light-dark cycle and free access to food and water. The experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and were approved by the local animal care committee.

3.2 Treatment and sample handling

MPTP was dissolved in phosphate-buffered saline (PBS; pH adjusted to 7.4) and was administered intraperitoneally (i.p.). Animals were randomly divided into six groups (n = 7-8 in each group). The first and second group received i.p. injection of 15 mg/kg body weight MPTP 5 times at 2 h intervals. The animals in the first group were deeply anesthetized with isoflurane (Forane; Abott Laboratories Hungary Ltd., Budapest, Hungary) and the brains were dissected ninety minutes following the last MPTP injection (acute treatment – acute (day 1) assessment), while animals in the second group were deeply anesthetized with isoflurane and the brains were dissected one week later (acute treatment - subacute (day 7) assessment). The mice in the third group were injected i.p. with 15 mg/kg body weight MPTP once a day for 12 days (low-dose chronic treatment). Ninety minutes following the last injection the animals

were euthanized via isoflurane overdose as well. The fourth, fifth and sixth groups served as the respective control groups, and were injected with 0.1 M PBS according to the above-detailed treatment regimen. During the dissection process the brains were rapidly removed on ice and immediately halved at the midline. Following that, both hemispheres were further cut to obtain the striatum, cortex and cerebellum. Thereafter, these samples were stored at -80^{o}C until the RT-PCR and HPLC analysis.

3.3 RT-PCR Analysis

The left striatum, cortex and cerebellum were homogenized and Trizol reagent was used to extract RNA according to the manufacturer's protocol. The RNA was quantified spectrophotometrically, and the integrity of RNA was confirmed by gel electrophoresis using 1% agarose gel. 1 μg of total RNA was reverse-transcribed applying random hexamer primers and reverse transcriptase according to the RevertAid First Strand cDNA Synthesis Kit protocol (Thermo Fisher Scientific Inc., Marietta, OH, USA). cDNAs were kept at -20^{o}C until further use.

Real-time PCR reactions were carried out in a 20 μl final volume.

The following, previously published primers were used: for FL-PGC-1α, 5'-TGCCATTGTTAAGACCGAG-3' (forward) and 5'-TTGGGGTCATTTGGTGAC-3' (reverse); for NT-PGC-1α, 5'-GGTCACTGGAAGATATGGC-3' (reverse); for CNS-PGC-1α and Ref-PGC-1α, 5'-AATTGGAGCCCCATGGATGAAGG-3' and 5'-TGAGTCTGTATGGAGTGACATCGAGTG-3' (both forward), and 5′-TCAAATGAGGGCAATCCGTC-3′ (reverse), respectively (Chang et al., 2012; Soyal et al., 2012). qRT-PCR reaction conditions were 95^{o}C for 2 min, followed by 40 cycles of 95^{o}C for 10 s, and 60^{o}C for 30 s. Target gene expression was normalized to the endogenous control

gene 18S rRNA (Applied Biosystems, Carlsbad, CA, USA). The relative expression was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

3.4 Dopamine measurement

DA and its metabolites, DOPAC and HVA were measured by reversed-phase chromatography from the right striatum of the MPTP-treated and the control animals, applying an Agilent 1100 high-performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA, USA) combined with a Model 105 electrochemical detector (Precision Instruments, Marseille, France) under isocratic conditions. The striata were weighted and then homogenized in an ice-cold solution (750 µl) containing perchloric acid (70% wt/wt), sodium metabisulfite (0.1 M), disodium ethylenediaminetetraacetate (0.1 M), distilled water and 0.25 mM isoproterenol for 30 sec. The homogenate was centrifuged at 12,000 g for 10 min at 4^{o}C. The supernatant was stored at -20^{o}C until the analysis. The supernatants were measured with an Agilent 1100 high-performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA, USA) combined with a Model 105 electrochemical detector (Precision Instruments, Marseille, France) under isocratic conditions. In brief, the working potential of the detector was set at +750 mV, using a glassy carbon electrode and an Ag/AgCl reference electrode. The mobile phase containing sodium dihydrogenphosphate (75 mM), sodium octylsulfate (2.8 mM) and disodium ethylenediaminetetraacetate (50 µM) was supplemented with acetonitrile (10% v/v) and the pH was adjusted to 3 with phosphoric acid (85% w/w). The mobile phase was delivered at a rate of 1 ml/min at 40°C onto the reversed-phase column (HR-80 C18, 80×4.6 mm, 3 µm particle size; ESA Biosciences, Chelmsford, MA, USA) after passage through a pre-column (SecurityGuard, 4 x 3.0 mm I.D., 5 µm particle size, Phenomenex Inc., Torrance, CA, USA)). 10 µl aliquots were injected by the autosampler with the cooling module set at 4°C. With regard to method validation, the following parameters are reported briefly. The LOD and LLOQ for the investigated

compounds in the brain samples were 2 ng/ml and 10 ng/ml, respectively. With regard to precision, the relative standard deviation was ≤ 3.25% for the peak area responses and ≤ 0.05% for the retention times. The recoveries ranged from 109 to 110%, 108 to 109% and 99 to 102% for DA, DOPAC and HVA, respectively.

3.5 Statistics

All statistical analyses were performed with the use of the R software (R Development Core Team, 2002). The distribution of data populations was checked with the Shapiro–Wilk test, and Levene test was also performed for the analysis of the homogeneity of variances. In case of gene expression analysis, due to the necessity of a large number of comparisons of data, two-sample t-tests *via* Monte-Carlo permutation (with 10,000 random permutations) were applied for RT-PCR results. In case of HPLC analysis, all the data exhibited normal distribution and equal variances were assumed, and therefore ANOVA was used with Bonferroni post hoc comparison. The null hypothesis was rejected when the corrected *p* values were < 0.05, and in such cases the differences were considered significant. FL- and NT-PGC-1α levels of gene expression of all brain areas were calculated relative to the levels of FL-PGC-1α gene expression in the striatum, whereas the CNS- and Ref-PGC-1α expression levels of all brain areas were calculated relative to the level of CNS-PGC-1α expression in the striatum. Data with Gaussian or non-Gaussian distributions were plotted as means (± S.E.M.) or medians (and interquartile range), respectively.

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Conflict of interest

The authors declare there is no conflict of interest.

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Figure legends

Fig. 1 The relative mRNA expression of PGC-1α isoforms in the striatum, cortex and the cerebellum of mice 90 min following acute MPTP intoxication. The FL-PGC-1α, NT-PGC-1α and CNS-PGC-1α levels were significantly increased in the striatum, cortex and the cerebellum of MPTP-treated mice. (A, B, C respectively). The Ref-PGC-1α expression did not change in any brain areas of MPTP-treated mice compared to the controls (D). Values are plotted as medians and interquartile range; *p < 0.05, **p < 0.01; *MPTP* MPTP-treated; *str* striatum, *ctx* cortex, *crb* cerebellum.

Fig. 2 The relative mRNA expression of PGC-1α isoforms in the striatum, cortex and the cerebellum of mice 7 days following acute MPTP intoxication. The expression levels of the PGC-1α isoforms did not change in any brain areas of MPTP-treated mice (A-D). Values are plotted as medians and interquartile range; *MPTP* MPTP-treated; *str* striatum, *ctx* cortex, *crb* cerebellum.

Fig. 3 The relative mRNA expression of PGC-1α isoforms in the striatum, cortex and the cerebellum of mice following a 12-day treatment with low-dose MPTP. The expression levels of the PGC-1α isoforms did not change in any brain areas of MPTP-treated mice (A-D). Values are plotted as medians and interquartile range; *MPTP* MPTP-treated; *str* striatum, *ctx* cortex, *crb* cerebellum.

Fig. 4 Striatal dopamine, DOPAC and HVA concentrations of MPTP-treated mice in 3 different treatment regimens. Ninety minutes (acute-1d) and 7 days (acute-7d) following acute MPTP intoxication, DA, DOPAC and HVA levels significantly decreased in the striatum compared to the controls. The chronic (12 day) low-dose MPTP treatment did not influence the striatal level of DA and DOPAC, only HVA levels were significantly decreased. Values

are plotted as means ± S.E.M; ***p < 0.001; *DA* dopamine, *DOPAC* 3,4-dihydroxyphenylacetic acid, *HVA* homovanillic acid