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ORIGINAL ARTICLE

Selective blockade of mGlu5 metabotropic glutamate receptors is protective against hepatic mitochondrial dysfunction in 6-OHDA lesioned Parkinsonian rats

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SUMMARY

Non-motor symptoms including those involving the splanchnic district are present in Parkinson's disease (PD). The authors previously reported that PD-like rats, bearing a lesion of the nigrostriatal pathway induced by the injection of 6-hydroxydopamine (6-OHDA), have impaired hepatic mitochondrial function. Glutamate intervenes at multiple levels in PD and liver pathophysiologies. The metabotropic glutamate receptor 5 (mGluR5) is abundantly expressed in brain and liver and may represent a pharmacological target for PD therapy. This study investigated whether and how chronic treatment with 2-methyl-6-(phenylethynyl)-pyridine (MPEP), a well-characterized mGluR5 antagonist, may influence hepatic function with regard to neuronal cell loss in PD-like rats. Chronic treatment with MPEP was started immediately (Early) or 4 weeks after (Delayed) intrastriatal injection of 6-OHDA and lasted 4 weeks. Early MPEP treatment significantly prevented the decrease in adenosine triphosphate (ATP) production/content and counteracted increased reactive oxygen species (ROS) formation in isolated hepatic mitochondria of PD-like animals. Early MPEP administration also reduced the toxin-induced neurodegenerative process; improved survival of nigral dopaminergic neurons correlated with enhanced mitochondrial ATP content and production. ATP content/production, in turn, negatively correlated with ROS formation suggesting that the MPEP-dependent improvement in hepatic function positively influenced neuronal cell survival. Delayed MPEP treatment had no effect on hepatic mitochondrial function and neuronal cell loss. Antagonizing mGluR5 may synergistically act against neuronal cell loss and PD-related hepatic mitochondrial alterations and may represent an interesting alternative to non-dopaminergic therapeutic strategies for the treatment of PD.

Key words: ATP production, liver, 2-methyl-6-(phenylethynyl)-pyridine, neuroprotection, Parkinson's disease.

INTRODUCTION

Parkinson's disease (PD) is characterized by the loss of dopaminergic nigrostriatal neurons that causes complex functional alterations in the basal ganglia (BG). Although PD is the most common movement disorder, numerous non-motor alterations are also present in patients and have been observed in animal models of the disease. Many of these non-motor symptoms affect the splanchnic district[1] and it has been proposed that the liver is one of the organs involved in the cascade of events following the central dopaminergic deficit.[2] Although in routine blood analysis PD patients do not usually present any significant alterations in hepatic enzyme levels, reports of impaired hepatic P450 subsystems, as well as reduced detoxification of medications and toxins,[3] clearly suggest that PD patients suffer some degree of liver dysfunction.

Parkinson's disease pathogenesis is linked to mitochondrial defects, which may cause reduced adenosine triphosphate (ATP) production and subsequent bioenergetic failure, as well as increased production of reactive oxygen species (ROS). We have recently demonstrated that PD-like rats, bearing a unilateral nigrostriatal lesion induced by the intrastriatal injection of the dopaminergic neurotoxin 6-hydroxydopamine (6-OHDA),[4] have altered hepatic mitochondrial functions compared to sham animals.[5] Previous data from the authors indicate that the neurotoxin only acts at the site of injection and that deficits observed in the liver can only be a consequence of alterations within the central nervous system (CNS). In PD-like rats, the extent of mitochondrial bioenergetic failure in the liver directly correlated with the degree of toxin-induced dopaminergic cell loss leading to the suggestion that the brain–liver–brain axis might be involved in a positive feedback mechanism in which subtle liver dysfunctions,

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arising from dopaminergic cell loss, may in turn further sustain the neurodegenerative process.

Glutamate mediates excitatory neurotransmission at crucial points in the BG and plays a central role in the mechanisms underlying motor symptoms and neurodegenerative processes in PD.[6] The neurotransmitter acts through G protein-coupled ionotropic (iGluRs) and metabotropic glutamate receptors (mGluRs); iGluRs mediate fast excitatory glutamatergic transmission, while mGluRs have a modulatory role and allow fine-tuning of glutamatergic transmission. Group I mGluRs, which include mGluR1 and mGluR5, are highly expressed in the BG where they regulate neuronal excitability and synaptic transmission. In the past decade, antagonizing mGluR5 in order to modulate the excessive glutamatergic transmission caused by the loss of dopaminergic innervation has attracted considerable attention.[7] 2-methyl-6-(phenylethynyl)-pyridine (MPEP) is a potent non-competitive mGluR5 antagonist that readily crosses the blood–brain barrier following systemic application.[8] MPEP can significantly improve akinesia and L-DOPA-induced abnormal involuntary movements (dyskinesias) in rodent as well as primate models of PD.[4,9–11] Preclinical *in vivo* data also indicate that chronic treatment with MPEP can reduce 6-OHDA-induced neurodegenerative processes in PD-like rats[12,13] suggesting that antagonizing mGluR5 may also have disease modifying potential. Two recently developed mGluR5 modulators, AFQ056 and AX48621, have demonstrated significant anti-dyskinetic activity in advanced PD patients with moderate-to-severe L-DOPA-induced dyskinesia,[14–16] further sustaining the use of mGluR5 antagonists for PD treatment.

The effects of mGluR5 antagonism on PD non-motor symptoms have not been evaluated so far. Evidence indicates that mGluR5 are also highly expressed in rat liver, in particular in hepatocytes, where their levels are comparable to those detected in the brain.[17] Interestingly, antagonizing mGluR5 with MPEP significantly reduces liver cell injury under hypoxic conditions[17] and hepatocytes isolated from mGluR5 knockout mice are less vulnerable to hypoxic cell damage.[18] MPEP can also reduce acetaminophen-triggered ROS production[19] and lipopolysaccharide-induced damage in mice livers.[20]

It is posited here that systemic treatment with MPEP could tune glutamatergic transmission at both ends of the brain–liver axis, thus synergistically modulating neuronal cell survival in the brain and hepatic function at the peripheral level and possibly modifying the brain-peripheral positive feedback loop triggered by neurodegenerative processes. To address this hypothesis, PD-like rats received a chronic, daily systemic injection of MPEP for a 4-week period. At the end of the treatment period, the effect of mGluR5 blockade on the extent of 6-OHDA-induced nigrostriatal degeneration and hepatic mitochondrial alterations were evaluated.

RESULTS

6-OHDA induced nigrostriatal lesions and effects of chronic MPEP administration

Animals received an intrastriatal injection of 6-OHDA (PD-like rats), or saline (sham) and were killed at different time-points (4 or 8 weeks) after the neurotoxic insult. A chronic systemic treatment with MPEP (1.5 mg/kg per day, intra-peritoneal (i.p.)) or vehicle was started immediately (Early) or 4 weeks after the neurotoxin injection (Delayed) (Fig. 1a).

Intrastriatal injection of 6-OHDA induced the typical retrograde neurodegeneration affecting tyrosine hydroxylase-positive (TH+) nigrostriatal neurons (Fig. 1b–d). A significant loss in TH+ terminals was detected in the striatum after 4 weeks (69.9 ± 2.9% which remained stable for 8 weeks (66.5 ± 7.2%) after neurotoxin injection (Fig. 1b). Similarly, a significant loss of TH+ neurons was observed in the SNc at 4 weeks (56.7 ± 6.2%) that remained stable for 8 weeks (51.9 ± 2.5%) after 6-OHDA injection (Fig. 1c,d).

Delayed treatment with the mGluR5 antagonist had no effect on toxin-induced nigrostriatal degeneration in the striatum (60.3 ± 4.3% vs 66.5 ± 7.2% terminal loss; Fig. 1b) and SNc (51.9 ± 5.9% vs 52.2 ± 2.5% cell loss; Fig. 1b–d). Similarly, early treatment with MPEP did not significantly modify the loss of TH+ terminals in the striatum (60.3 ± 6.2% vs 69.9 ± 2.9% terminal loss; Fig. 1b). Conversely, a significant rescue of TH+ neurons in the SNc was observed when the chronic treatment with MPEP was started immediately after the neurotoxic insult (56.7 ± 6.2 vs 45.4 ± 4.5 cell loss; Fig. 1c,d).

Effect of MPEP administration on liver enzyme activities in 6-OHDA-treated rats

Levels of liver enzymes, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), phosphatase alkaline (PA) and lactate dehydrogenase (LDH), in the blood can be used to assess the presence of liver dysfunction or injuries. PD-like rats, killed 4 and 8 weeks after 6-OHDA injection, showed normal AST, ALT, PA and LDH levels. Interestingly, early chronic MPEP treatment, started at the time of the 6-OHDA injection, significantly reduced ALT levels (Fig. 2a) compared to those observed in PD-like rats receiving no treatment. No effect was observed when MPEP treatment was delayed (Fig. 2c,d).

Chronic MPEP administration improves hepatic mitochondrial bioenergetic capacity

Mitochondrial membrane potential ($\Delta\Psi$) of liver mitochondria obtained from PD-like rats 4 weeks after 6-OHDA infusion was comparable to that measured in sham animals and was not modified by early MPEP treatment (-103.0 ± 2.0 mV vs -105.4 ± 5.9 mV; ANOVA $F_{3,29} = 2.292$, $P < 0.01$). A significant decrease in hepatic mitochondrial $\Delta\Psi$ was detected in PD-like rats 8 weeks after the neurotoxin injection as compared to sham animals (-94.4 ± 3.7 mV vs -118.2 ± 2.8 mV, ANOVA $F_{3,28} = 12.05$, $P < 0.001$, $P = 0.0006$ 6-OHDA vs SHAM) but this effect was not modified by delayed MPEP treatment.

Liver mitochondria obtained from PD-like rats, sacrificed 4 or 8 weeks after 6-OHDA intrastriatal injection, exhibited a significant decrease in ATP production and ATP content, as compared to the corresponding sham animals (Fig. 3a,b). Early chronic administration of MPEP significantly increased ATP production and content in liver mitochondria obtained from PD-like animals (Fig. 3a). Conversely, delayed MPEP treatment had no effect on the toxin-dependent decrease in ATP content and production (Fig. 3b). Chronic treatment with MPEP in sham animals had no effect on liver mitochondrial ATP levels.

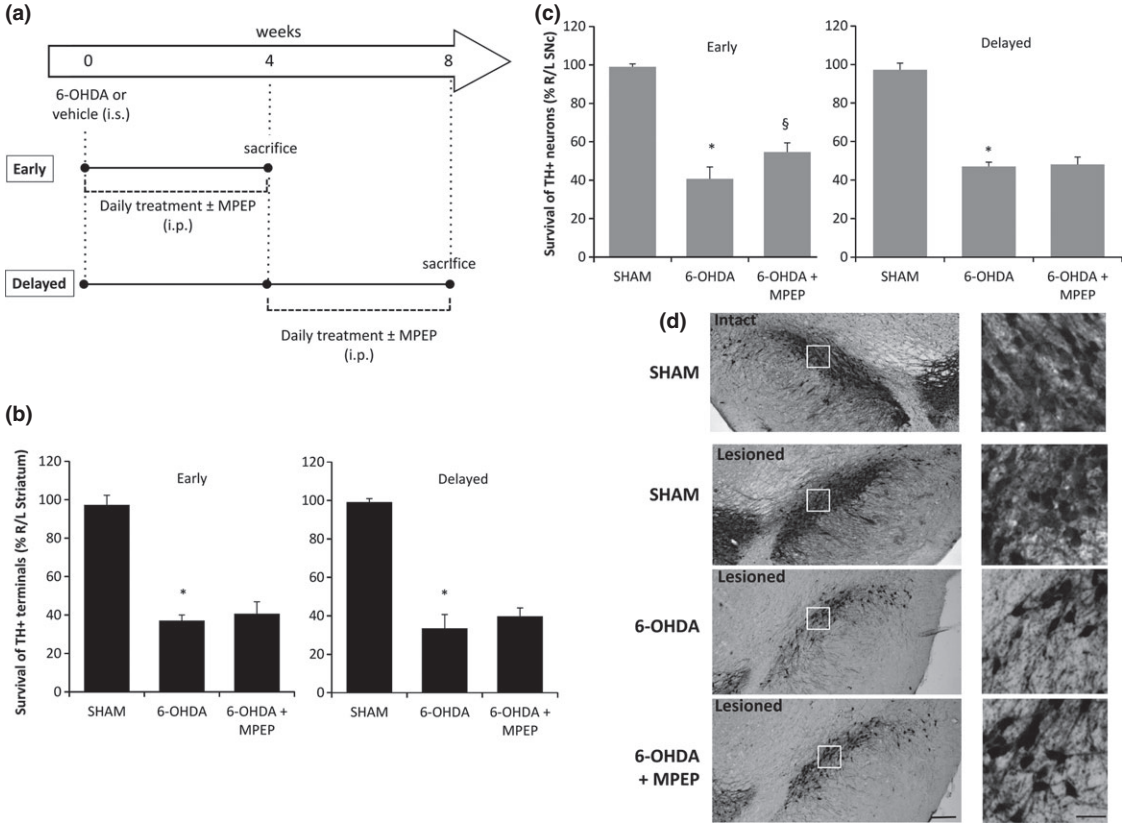

Fig. 1 MPEP (2-methyl-6-(phenylethynyl)-pyridine) treatment and nigrostriatal degeneration. (a) Schematic representation of the experimental design. Stereotaxic intrastriatal (i.s.) injection of 6-hydroxydopamine (6-OHDA), or vehicle, was carried out at time 0, for all groups. Lesioned (6-OHDA) or sham (vehicle) animals were treated daily with MPEP (1.5 mg/kg, i.p.), or vehicle, for 4 weeks. Treatment was started immediately after surgery (Early) or 4 weeks later (Delayed). Animals were killed at the end of the 4-week treatment, which corresponded to 4 weeks (for Early treatment) or 8 weeks (for Delayed treatment) post-6-OHDA injection. (b) Effect of MPEP treatment on striatal dopaminergic terminals. Chronic injection of the mGluR5 antagonist was started immediately after (Early) or 4 weeks (Delayed) after injection of 6-OHDA. Results represent the percentage (mean ± SE) of striatal TH+ terminals in the lesioned hemisphere (R) compared to the intact hemisphere (L). ANOVA (Early) $F_{3,18} = 35.33$ $P < 0.0001$, $^{*}P < 0.0001$ versus sham; ANOVA (Delayed) $F_{3,14} = 39.45$ $P < 0.0001$, $^{*}P < 0.0001$ versus sham. (c) Effect of chronic MPEP treatment on dopaminergic neurons in the SNc. Chronic injection of the mGluR5 antagonist was started immediately after (Early) or 4 weeks (Delayed) after injection of 6-OHDA. Results represent the percentage (mean ± SE) of TH+ neurons in the lesioned SNc (R) compared to the intact hemisphere (L); ANOVA (Early) $F_{3,18} = 37.90$, $P < 0.0001$, $^{*}P < 0.001$ versus sham, $^{\S}P = 0.0388$ versus 6-OHDA ANOVA (Delayed) $F_{3,14} = 96.36$, $P < 0.0001$, $^{*}P < 0.001$ versus sham. (d) Representative photomicrographs of brain coronal sections showing TH+ cells in the SNc of the intact and lesioned hemispheres (as indicated) in sham, 6-OHDA and 6-OHDA+ MPEP animals killed 4 weeks after 6-OHDA injection. Lower (left panels; scale bar = 200 μm) and higher magnifications (right panels; scale bar = 50 μm) are shown.

Increased production of hepatic mitochondrial ROS was detected in PD-like animals compared to sham animals (Fig. 4). A significant increase in ROS production was detected only 8 weeks after the toxic insult. These results are similar to those observed for $\Delta\Psi$ and suggest that increased ROS production arises as a consequence of sustained neuronal cell loss and mitochondrial bioenergetic alterations. When chronic treatment with MPEP was started immediately after the 6-OHDA injection (Early), a significant reduction in the 6-OHDA-dependent increase in ROS production was observed in PD-like animals, as compared to the corresponding untreated rats and sham animals (Fig. 4).

The investigation then looked at how liver mitochondrial ATP content/production and ROS production related to each other as well as to the number of TH+ neurons in the SNc of the lesioned hemisphere. We considered all the animals, sham and 6-OHDA, in terms of presence and absence of MPEP treatment. The results indicate that higher hepatic mitochondrial energy capacity corresponds to a higher number of surviving dopaminergic neurons in the SNc and decreases in parallel with the loss of TH+ neurons in the SNc (Fig. 5a), suggesting a link between liver mitochondrial energy capacity and nigral cell survival. In parallel, a moderate negative correlation between ATP content or production, and the propensity to produce ROS in hepatic mitochondria (Fig. 5b) was observed, indicating that a sustained hepatic mitochondrial ATP deficit may drive increased ROS production.

DISCUSSION

The authors' previous data[5] indicated that PD-like rats exhibit significant hepatic mitochondrial alterations that develop parallel to nigrostriatal neurodegeneration, suggesting the existence of a complex brain–liver functional relationship, as already described for the brain-gut axis.[21,22] In the present study, we have assessed

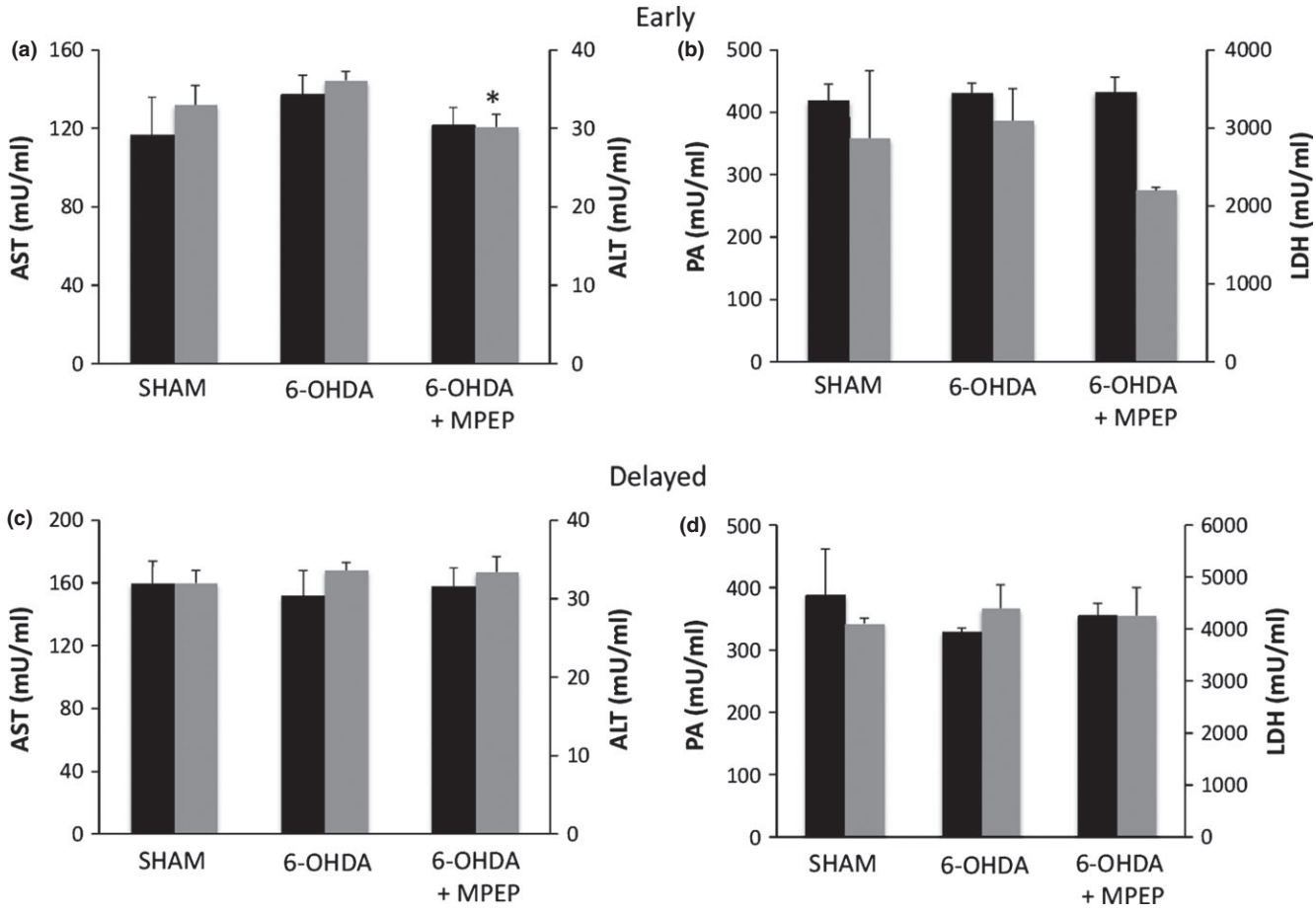

Fig. 2 MPEP (2-methyl-6-(phenylethynyl)-pyridine) administration and serum levels of hepatic enzymes. Effect of Early (a, b) and delayed (c, d) MPEP treatment on serum levels of aspartate aminotransferase (AST) and ALT (a, c), as well as PA and LDH (b, d) in 6-hydroxydopamine (6-OHDA) or SHAM animals. Values represent the mean ± SE of 4–6 animals. ANOVA: AST early $F_{3,19} = 0.6882$, $P = 0.5703$; AST delayed $F_{3,12} = 0.1797$, $P = 0.9801$; ALT early $F_{3,19} = 3.549$, $P = 0.0341$, $^*P = <0.05$ versus 6-OHDA; ALT delayed $F_{3,12} = 1.167$, $P = 0.3630$; PA early $F_{3,19} = 0.096$, $P = 0.9614$; PA delayed $F_{3,12} = 0.9434$, $P = 0.045$; LDH early $F_{3,19} = 1.473$, $P = 0.2537$; LDH delayed $F_{3,12} = 0.4798$, $P = 0.7024$. ■, AST; ■, ALT; ■, PA; ■, LDH.

the effect of a chronic, systemic treatment with the mGluR5 antagonist MPEP on neuronal cell loss, and liver mitochondria deficits that develop as a consequence of the intrastriatal injection of the neurotoxin 6-OHDA.

The data from this study show that the peripheral treatment with MPEP can act at two levels. In the brain, it is confirmed that systemic and chronic treatment with MPEP, initiated immediately after neurotoxin-injection (Early), can significantly improve survival of dopaminergic neurons in PD-like rats with a unilateral 6-OHDA-induced lesion of the nigrostriatal pathway.[12,13] In the liver, it is demonstrated that the MPEP-dependent neuroprotection coincides with a recovery in ATP content and production, as well as in a time-dependent reduction of ROS production in mitochondria isolated from MPEP-treated PD-like animals.

The liver exerts a crucial role in preventing the accumulation of endogenous and exogenous toxins by promoting their clearance and avoiding their entry into the CNS. The liver may therefore be an important participant in a potential vicious circle in which (i) loss of dopaminergic neurons induces subtle hepatic dysfunctions, through mechanisms that still need to be clarified, and (ii) hepatic dysfunctions, in turn, further promote and sustain ongoing neurodegenerative processes. Critical hepatic conditions, such as liver failure and cirrhosis, can lead to severe encephalopathy that presents altered neurotransmitter levels, including glutamate.[23] Glutamate is an important player in the regulation of ammonia turnover and oxidative stress, and is critical in the pathophysiological liver–brain relationship.[24] Similarly, glutamate is essential in the modulation of excitatory neurotransmission and in the mediation of neuroinflammatory processes in astrocytes and microglia. The regulation of glutamate levels through the modulation of mGluR5 simultaneously in the brain and liver may represent a bilateral therapeutic strategy that will reduce or slow down neurodegenerative processes by acting at both ends of the brain-liver axis.

It was observed that, in isolated liver mitochondria, ROS production negatively correlates with ATP content and production, which in turn correlates with the toxin-induced loss of TH+ neurons in the SNc. Viewed in their entirety, the data presented here indicate that animals with the most well-preserved hepatic mitochondrial function are also those exhibiting a better preserved nigrostriatal pathway and *vice versa*. Overall protection may therefore be due, at least in part, to a reduction in ROS formation in the brain and liver.

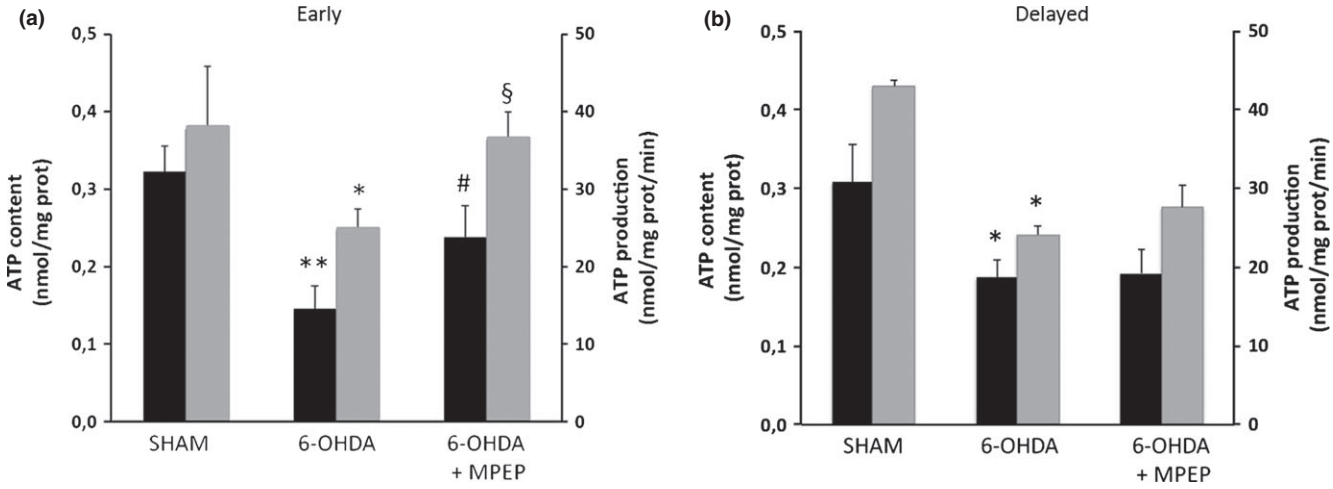

Fig. 3 MPEP (2-methyl-6-(phenylethynyl)-pyridine) treatment and adenosine triphosphate (ATP) content/production in freshly isolated liver mitochondria. (a) Effect of early MPEP treatment. Animals received chronic treatment with MPEP (1.5 mg/kg per day, i.p.), or vehicle, that was started immediately (Early) after intrastriatal injection of the neurotoxin injection, and were killed 4 weeks later. Hepatic mitochondria were isolated immediately and ATP content and production were measured. Values represent the mean ± SE of single mitochondria preparations obtained from individual animals (n = 4–6). Each measurement was performed in duplicate. ANOVA: ATP content $F_{3,25} = 7.182$ $P = 0.0012$, $^{**}P < 0.001$ versus sham, $\#P < 0.05$ versus 6-hydroxydopamine (6-OHDA); ATP production $F_{3,25} = 3.685$ $P = 0.0367$, $^{*}P < 0.05$ versus sham, $\S P < 0.02$ versus 6-OHDA. (b) Effect of delayed MPEP treatment. Animals received chronic treatment with MPEP (1.5 mg/kg per day, i.p.), or vehicle, that was started 4 weeks (Delayed) after intrastriatal injection of the neurotoxin injection, and were sacrificed after a 4-week period of treatment. Values represent the mean ± SE of single mitochondria preparations obtained from individual animals (n = 4–6). Each measurement was performed in duplicate. ANOVA: ATP content $F_{3,25} = 3.707$ $P = 0.0368$, $^{*}P < 0.05$ versus sham; ATP production $F_{3,25} = 13.07$ $P < 0.0001$, $^{*}P < 0.0001$ versus sham. ■, content; ■, production.

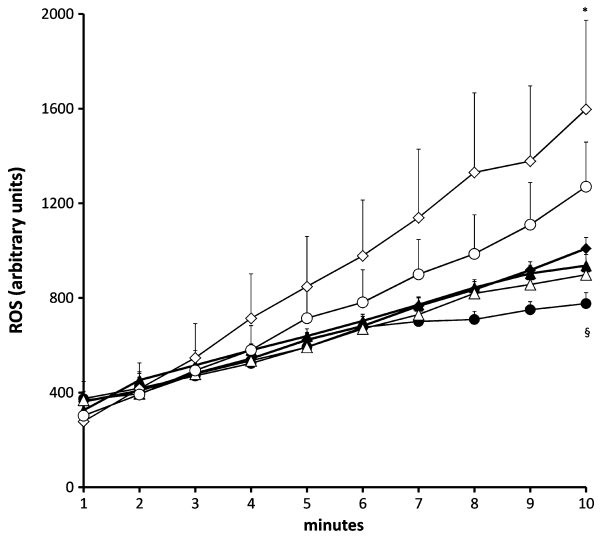

Fig. 4 Effect of chronic MPEP (2-methyl-6-(phenylethynyl)-pyridine) administration on hepatic mitochondrial reactive oxygen species (ROS) production. Hepatic mitochondria were isolated immediately at the time of sacrifice and ROS production was measured every minute over a 10-min period. Results (mean ± SE) are from mitochondria preparations obtained from individual PD-like animals (n = 4–6/group). Significant ROS production was observed at the end of the 10-min period. ANOVA: ROS early $F_{3,25} = 3.119$ $P < 0.05$, $\S P < 0.05$ versus 6-OHDA animals; ROS Delayed $F_{3,25} = 3.019$ $P < 0.05$, $^{*}P < 0.05$. –▲–, sham early; –◆–, 6-OHDA early; –●–, 6-OHDA MPEP early; –△–, sham delayed; –◇–, 6-OHDA delayed; –○–, 6-OHDA MPEP delayed.

Owing to their high reactivity, ROS are designated as signalling molecules in a variety of pathological processes. Recent data suggest that ROS can be released into the cytosol and further trigger ROS-induced ROS release in neighboring mitochondria.[25] Mitochondrial dysfunction and ROS-mediated cytotoxicity is known to play an important role in PD pathogenesis[26] and ROS may participate, in a concentration-dependent manner, in different processes involved in the disorder, including the regulation of pro-inflammatory cytokines, apoptosis and autophagy.[27] At the central level, injection of 6-OHDA causes the generation of ROS that contributes to the cascade of events leading to neuronal cell death.[28] Recent data have shown that, in a rodent model of PD induced by rotenone injection, neurodegeneration is accompanied by mGluR5-triggered downstream cellular pathways as well as ROS generation.[29] These effects could be blocked by pre-treatment with MPEP both in vitro and in vivo, indicating that antagonizing mGluR5 can reduce ROS production accompanying neurodegeneration. At the peripheral level mGluR5 are also highly expressed in hepatocytes[30] where they have been shown to play an important function in liver susceptibility to damage. Storto and collaborators have shown that MPEP provides cytoprotection and reduces ROS production in experimental liver damage induced by oxidative stress.[19] The authors first reported that, in hepatocyte cultures, addition of MPEP or SIB-1893, two mGluR5 antagonists, reduces tert-Butyl-hydroperoxide-induced cellular toxicity and ROS production.[19] MPEP treatment can also prevent acetaminophen-induced liver damage and ROS formation in mice and significantly decreases hepatic liver peroxidation in a model of fulminant hepatic failure induced by lipopolysaccharide and D-Galactosamine.[20] Interestingly, hepatocytes from mGluR5-knock-out mice show reduced sensitivity to hypoxic damage associated with reduced ROS generation.[18] Hence the overall protection reported in this study might be due, at least in part, to a reduction in mitochondrial ROS formation and propagation, and might further indicate that MPEP can positively modulate toxin-dependent alterations present at both ends of the brain-liver axis.

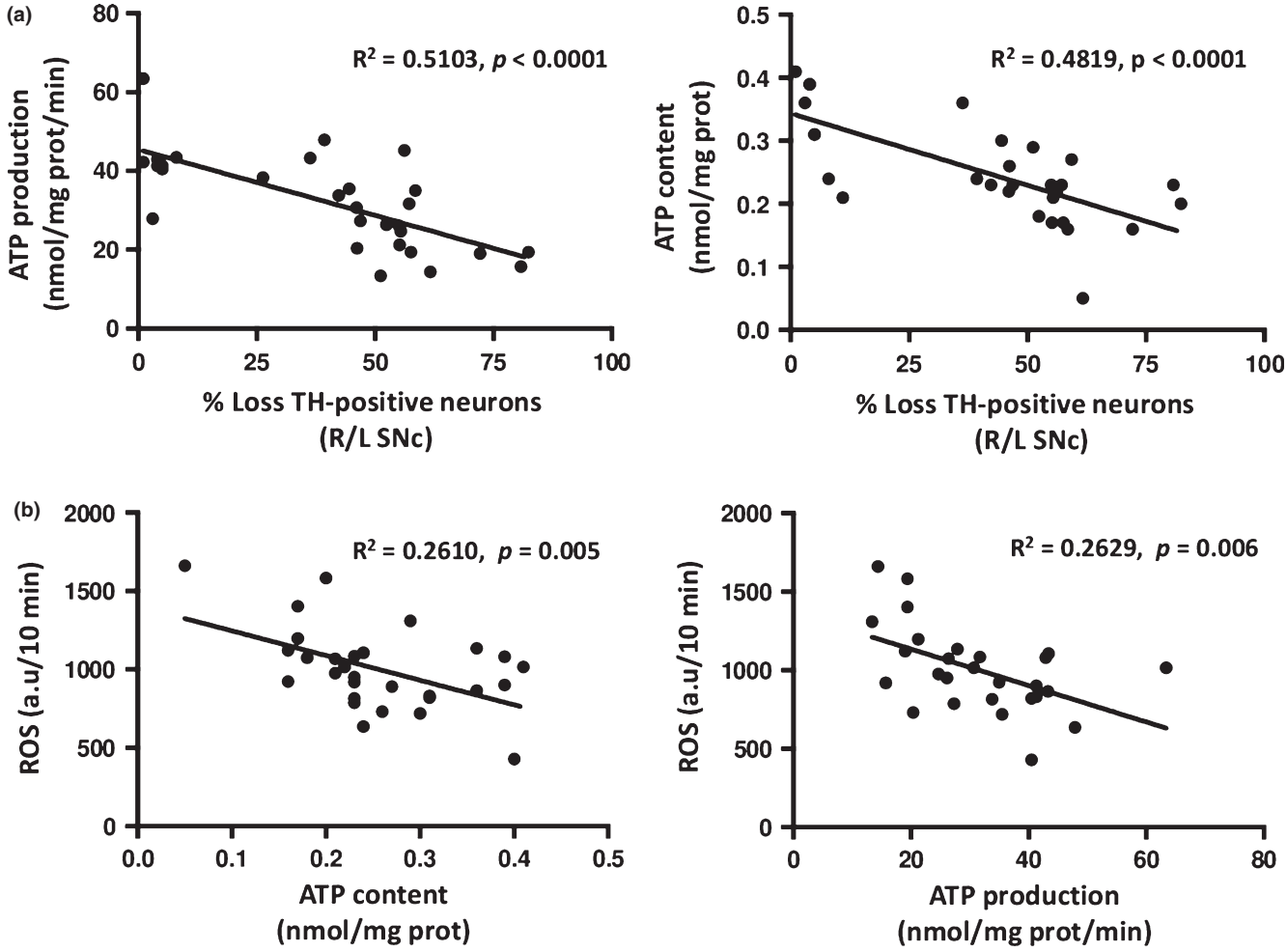

Fig. 5 Neuronal cell loss, adenosine triphosphate (ATP) content and production and reactive oxygen species (ROS) production. (a) Moderate correlation between loss of dopaminergic neurons in the SNc and ATP content (left panel) and production (right panel) of hepatic mitochondria indicating that reduced hepatic mitochondria bioenergetics correlates with increased neuronal cell loss in the SNc. (b) Correlation between ROS production and ATP content (left panel) and ATP production (right panel) in isolated liver mitochondria. Highest ROS production correlates with reduced ATP content or production.

Interestingly, delayed MPEP treatment had no influence on the neurodegenerative process and on parallel dysfunctions in liver mitochondria. This is in keeping with the multifactorial pathophysiology of PD, in which an initial pathogenic stimulus may trigger a cascade of events and compensatory mechanisms that evolve and change parallel to neurodegenerative processes as the disease progresses. In this scenario a delayed intervention on ROS generation might not be sufficient to slow down or reverse ongoing degenerative processes.

Visceral information is conveyed to the CNS via vagal afferents through the *nucleus tractus solitarius* to the dorsal motor nucleus of the vagus (DMV). The DMV receives glutamatergic inputs[31] and expresses mGluR that have some physiological relevance in the control of homeostatic alterations in peripheral organs and in the CNS. A recent study has demonstrated that 6-OHDA-induced nigrostriatal degeneration is accompanied by neurochemical alterations in the DMV that significantly impact on visceral physiology.[22] In our study, we observed a significant protection of both TH+ neurons and liver mitochondria when MPEP treatment was started early. The peripheral protective effect could be due to an MPEP-mediated, fine-tuned regulation of vagal outputs as well as to a local inhibition of hepatic mGluR5. Both mechanisms could act separately or in synergy to positively impact on liver dysfunction that emerges as a consequence of neuronal cell loss.

This study does not establish whether improved hepatic mitochondrial function positively affects the survival of the nigrostriatal pathway or *vice versa*. Hence, further studies are needed to establish whether alterations at central and peripheral levels are interconnected and whether greater or even synergistic benefits may be obtained with a therapeutic strategy that simultaneously acts at both ends of the brain-liver axis.

METHODS

Animals, surgery, sacrifice, tissue processing and Image analysis

Male Sprague–Dawley rats (Charles River, Wilmington, MA, USA), weighing 200–225 g at the beginning of the experiment,

were housed two per cage, at 20–22°C on a 12 h light–dark cycle, with food and water *ad libitum*. Animals were left in the housing facilities for at least 1 week, before the beginning of the experiments. All procedures were in accordance with the Italian guidelines for the care and use of experimental animals (DL 116/92) and European Communities Council Directive (2010/63/EEC) and were approved by the Animal Care Committee of the University of Pavia. Efforts were made to minimize the number of animals used.

Rats ($n = 40$; $n = 4$–6 per group) were anaesthetized with sodium-thiopental (50 mg/kg) and placed in a stereotaxic frame (Stoelting, Wood Dale, IL, USA) with the incisor bar positioned 3.3 mm below the interaural line. Animals received a unilateral injection of 6-OHDA (20 mg/3 μL in saline/0.02% ascorbic acid; Sigma-Aldrich, St Louis, MO, USA) or vehicle into the right striatum (1.0 mm anterior, 3.0 mm lateral and 5.0 mm ventral, with respect to bregma and dura) at 1 mL/min using a Hamilton 10 μL syringe with a 26-gauge needle. The needle was left in place for 5 min before being retracted to allow complete diffusion of the medium and wounds were clipped.[32] At the end of the treatment period, corresponding to a 4- or 8-week time lapse from the intrastriatal injection, animals were killed by decapitation and trunk blood was collected. The brains were removed and used for assessment of toxin-induced nigrostriatal lesions using Western blotting and immunohistochemistry as described before.[5,12] The brains were separated in two portions containing the striatum or the SNc using a brain coronal matrix. Striata were dissected out, homogenized and analyzed by Western blot analyses using a mouse anti-TH primary antibody (Chemicon (Millipore), Billerica, MA, USA) and IRDye800 or IRDye700 secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA). Striatal TH expression was quantified using an Odyssey Infrared Imaging System (LI-COR Biosciences). Samples were normalized to actin levels; results represent the percentage of TH+ terminal in the lesioned versus intact striatum. The brain portion containing the SNc was cut into 25 μm serial coronal sections using a cryostat and mounted on a polylysine-coated slide. Immunohistochemical staining was carried out using a mouse anti-TH primary antibody (Chemicon) and a biotinylated anti-mouse IgG secondary antibody (Vector Laboratories, Burlingame, CA, USA). Sections were processed with the avidin–biotin technique using a commercial kit (Vectastain ABC Elite kit; Vector Laboratories) and reaction products were developed using a nickel-intensified 3′-3′-diaminobenzedine tetra-hydrochloride (DAB Substrate Kit for Peroxidase; Vector Laboratories). The number of TH+ neurons in the SNC was counted bilaterally on every fourth section throughout the SNc using the unbiased stereological optical fractionator method (Stereo Investigator System; Microbrightfield Inc., Willston, VT, USA). Results represent the percentage of TH+ neurons in the lesioned SNc with respect to the intact hemisphere.

Isolation of liver mitochondria

Liver from each animal was removed immediately after sacrifice and processed for isolation of the mitochondrial fraction. Whole livers (9 g) were washed with ice-cold saline and processed immediately for mitochondria isolation using standard differential centrifugation.[25] Minced tissue was homogenized in an ice cold medium containing 0.25 mol/L sucrose, 1 mmol/L EDTA, 5 mmol/L HEPES (pH 7.2; all from Sigma) using a teflon/glass Potter homogenizer (Sartorius, Göttingen, Germany). The homogenate was centrifuged at 1000 *g* for 10 min. The supernatant was taken up and centrifuged again for 10 min at 10 000 *g*. The resulting pellet, was resuspended in a medium containing 0.25 mol/L sucrose, 5 mmol/L HEPES and centrifuged again for 10 min at 10 000 *g*, and kept on ice for subsequent determination (see below). Mitochondrial preparations were obtained individually from each single animal ($n = 4$–6) and protein concentrations were determined using the Lowry method.[26,33]

Determination of hepatic mitochondrial ATP content and production

The ATP content and ATP production were measured using the luminescence-based ATP-Lite kit (Perkin Elmer, Waltham, MA, USA) following the manufacturer's instructions. The production of luminescence caused by the reaction of ATP with Luciferase and D-Luciferin was detected using a Perkin Elmer Victor 2 luminometer.

Hepatic mitochondria pellets, obtained from individual animals, were resuspended in a phosphate buffer (250 mmol/L sucrose, 5 mmol/L KH_2PO_4 and 1 μmol/L rotenone, pH 7.2 at 25 °C) and each preparation was split into two. One half was lysed immediately to determine basal ATP content.[27,34] The second half was incubated with 25 μmol/L ADP and 6 mmol/L succinate, and ATP content was measured after 2 min. ATP production was determined as the difference between the basal ATP content and the ATP content after incubation with the substrates.[27,34] Results represent mean ± SE of ATP (nmol) produced per mg of protein.

Determination of hepatic mitochondrial reactive oxygen species

The generation of ROS from individual hepatic mitochondria preparations, obtained from individual animals, was evaluated by measuring the conversion of 2′,7′-dichlorofluorescein diacetate (H2DCFDA) to fluorescent 2′,7′-dichlorofluorescein (DCF). Freshly isolated rat mitochondria were re-suspended in 2 mL of phosphate buffer (250 mmol/L sucrose, 5 mmol/L KH_2PO_4, 1 μmol/L rotenone, pH 7.2 at 25 °C) and incubated for 10 min at 37 °C with 6 mmol/L succinate and 5 mmol/L H2DCFDA (Molecular Probes Inc., Carlsbad, CA, USA). Production of the fluorescent derivative DCF as a function of time (minutes) was measured using a microplate reader (Perkin Elmer Life Science, Monza, Italy).

Determination of hepatic mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi$) was assessed by measuring the uptake of the fluorescent dye rhodamine 123.[28,35] Measures were taken from single hepatic mitochondrial preparation obtained from individual animals and resuspended in 2 mL of phosphate buffer (250 mmol/L sucrose, 5 mmol/L KH_2PO_4, 1 μmol/L rotenone, pH 7.2 at 25 °C) containing 6 mmol/L succinate, and 0.3 μmol/L rhodamine 123. The fluorescence of rhodamine 123 was monitored using a Perkin Elmer LS 50B fluorescence spectrometer. Excitation and emission wavelengths

were 503 and 527 nm, respectively. $\Delta\Psi$ (mV) was calculated according to the following relationship: $\Delta\Psi = -59$ log (rhodamine 123) in/(rhodamine 123) out, assuming that the distribution of rhodamine 123 between mitochondria and medium follows the Nernst equation. Each measurement was performed twice in duplicate immediately after sacrifice on fresh mitochondria preparations. Due to possible inter assay differences only animals from the same experimental paradigm, early or delayed, were directly compared in the statistical analyses.

Serum levels of hepatic enzymes

Evaluation of liver transaminases (alanine aminotransferase (ALT), aspartate aminotransferase (AST)) phosphatase alkaline (PA) and lactate dehydrogenase (LDH) was performed on rat serum using an automated Hitachi 747 analyzer (Roche/Hitachi, Hitachi Ltd., Chiyoda, Tokyo, Japan).

Statistical analysis

All values are expressed as mean ± SE. Comparisons among groups were carried out using the analysis of variance (ANOVA) coupled with the Tukey's *post-hoc* correction using a dedicated software (PRISM 3 software; GraphPad, La Jolla, CA, USA). Correlation among variables was assessed by the Pearson's correlation coefficient (r). The minimum level of statistical significance was set at $P < 0.05$. MPEP treatment *per se* in sham animals had no effect on any of the variables assessed in this study. Sham and sham + MPEP groups were considered separately for the statistical analysis but were pooled for the representation of the data as graphs.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

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