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Lack of involvement of glutamate-induced excitotoxicity in MPP^+ toxicity in striatal dopaminergic terminals: possible involvement of ascorbate

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1 The present study concerns the possible relationship between glutamate excitotoxicity and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine/1-methyl-4-phenylpyridinium (MPTP/MPP^+) neurotoxicity on striatal dopaminergic terminals.

2 MPP^+ neurotoxicity has been studied by means of two MPP^+ perfusions separated by 24 h. After the second MPP^+ 1 mM perfusion, dopamine extracellular output, measured by microdialysis, was considered to be an index of the dopaminergic neurone damage produced by the first MPP^+ 1 mM perfusion.

3 High concentration (10 mM) of glutamate uptake inhibitor L-trans-pyrrolidine-2,4-dicarboxylic acid (PDC) stimulated basal release of dopamine and protected against the neurotoxic effect of MPP^+.

4 PDC 10 mM perfusion produced an increase in the extracellular output of glutamate and aspartate, and a decrease in that of ascorbate.

5 The protective effect against MPP^+ toxicity observed with PDC 10 mM was completely abolished when this glutamate uptake inhibitor was co-perfused with ascorbate 0.5 mM.

6 These results suggest that glutamate-induced neurotoxicity is not involved in MPP^+ toxicity. The protective effect found with the glutamate uptake inhibitor could be due to a decrease in extracellular ascorbate levels.

Keywords: Dopamine release; glutamate uptake; ascorbate; 1-methyl-4-phenylpyridinium; microdialysis; rat striatum

Introduction

Degeneration of dopaminergic nigrostriatal neurones with depletion of striatal dopamine is the primary histopathological finding in Parkinson's disease. The cause of the neurodegenerative process leading to loss of these dopaminergic neurones is still unknown. However, hypotheses implicating free radicals, including those generated by metabolism of dopamine (Riederer & Youdim, 1986) and excitotoxins (Zuddas *et al*., 1992) in the death of dopaminergic neurones have been proposed. The discovery of the selective nigrostriatal toxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has increased the knowledge of the possible mechanisms that could be involved in this neurodegenerative process. MPTP-induced mitochondrial dysfunction at the level of the complex I rotenone binding site (Ramsay *et al*., 1991) results in intracellular adenosine 5′-triphosphate (ATP) depletion (Chan *et al*., 1991). Other events that may contribute to cell death include disturbance of cytosolic Ca^{2+} homeostasis, perturbation of cytoskeletal organization, generation of free radicals and lipid peroxidation, and potentiation of endogenous excitatory amino acid action.

The concept of excitotoxicity (i.e., neuronal damage resulting from excessive activation of postsynaptic excitatory receptors) was first proposed to explain the selective neuronal death that glutamate or other neuroexcitatory compounds can produce (Olney, 1990). The notion that this excitotoxic mechanism was involved in MPP^+ toxicity was based on the observation that intrastriatal administration of MPP^+ can lead to a massive rise in extracellular glutamate and aspartate (Carboni *et al*., 1990). Subsequent support came from a study describing protective effects of excitatory amino acid receptor antagonists in rats given an intranigral infusion of MPP^+ (Turski *et al*., 1991). However, later studies with glutamate antagonists have yielded mixed results, with accounts of full protection (Storey *et al*., 1992; Srivastava *et al*., 1993), partial protection (Santiago *et al*., 1992; Chan *et al*., 1993; Brouillet & Beal, 1993) or no protection (Sonsalla *et al*., 1989; 1992; Kupsch *et al*., 1992; Finiels-Marlier *et al*., 1993). Recently, Venero *et al*. (1996) have suggested that the partial protection produced by MK-801 against MPP^+ could be produced by its inhibitory effect on the dopamine uptake system.

The purpose of this study was to collect detailed information on the time course of changes in MPP^+ neurotoxicity on dopaminergic terminals in rat striatum with increasing extracellular glutamate concentration. The increase in extracellular glutamate concentration was specifically achieved by inhibiting glutamate uptake with L-*trans*-pyrrolidine-2,4-dicarboxylic acid (PDC) (Bridges *et al*., 1991). There is some evidence indicating that ascorbate is released by heteroexchange at the glutamate transporter (Fillenz & Grünewald, 1984; Rebec & Pierce, 1994) and that physiologically evoked release of ascorbate is inhibited by glutamate uptake inhibitors (Miele *et al*., 1994). The concentration of PDC used in these experiments was able to produce an increase in glutamate/aspartate levels and a significant decrease in extracellular ascorbate levels.

Methods

Animals

Animals were male albino Wistar rats weighing 270–320 g at the time of probe implantation. The rats were kept, three or four rats per cage, at constant room temperature ($22 \pm 2°C$) and relative humidity (60%) with a 12 h light-dark cycle and unlimited access to food and water. Experiments were carried out in accordance with the Guidelines of the European Union Council (86/609/EU), following the Spanish regulations (BOE 67/8509-12, 1988) for the use of laboratory animals and approved by the Scientific Committee of the University of Sevilla.

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Surgery and brain dialysis

Microdialysis in the corpus striatum was performed with an I-shaped cannula (Santiago & Westerink, 1990). The exposed tip of the dialysis membrane was 4 mm. The dialysis tube (i.d.: 0.22 mm; o.d.: 0.31 mm) was prepared from polyacrylonitrile/sodium methalyl sulphonate copolymer (AN 69, Hospal, Bologna, Italy). The probe was stereotaxically implanted into both corpus striata with coordinates from bregma point and dura (A/P + 0.6, L/M 2.8, V/D 6.0; Paxinos & Watson, 1986), during general chloral hydrate (400 mg kg^{-1}, i.p.) and local lidocaine (10% w/v in water) anaesthesia. Following surgery, animals were housed individually in plastic cages ($35 \times 35 \times 40$ cm).

The perfusion experiments were carried out 24–48 h after implantation of the probe. Microdialysis and subsequent chemical analysis of dopamine and ascorbic acid were performed with an automated on-line sample injection system (Westerink *et al*., 1987). The corpus striatum was perfused at a flow rate of 3.0 μl min^{-1}, by use of a microperfusion pump (model 22, Harvard Apparatus, South Natick, MA, U.S.A.), with a Ringer solution containing (in mM): NaCl 140, KCl 4.0, $CaCl_2$ 1.2 and $MgCl_2$ 1.0. With the help of an electronic timer, the injection valve was held in the load position for 15 min, during which time the sample loop (40 μl) was filled with dialysate. The valve then switched automatically to the injection position for 15 s. This procedure was repeated every 15 min, which was the time needed to record a complete chromatogram. For the measurement of aspartate and glutamate, dialysates were collected every 30 min and derivatized immediately before their injection into the high performance liquid chromatography (h.p.l.c.) valve. After a steady baseline of levels had been established in four consecutive samples (in fmol min^{-1}), drugs were administered and sampling was continued for 2.5 h thereafter. All drugs were dissolved in Ringer solution and when necessary, pH was adjusted to 6.0 with NaOH 0.1 M.

Twenty-four hours after surgery (day 1), one group of experiments was carried out with rats perfused with MPP^+ 1 mM for 15 min and other groups with rats perfused with PDC, ascorbic acid (AA) or PDC + AA for 1 h followed by a 15 min co-perfusion with MPP^+ 1 mM.

Forty-eight hours after surgery (day 2), MPP^+ 1 mM was perfused in every group for 15 min.

At the end of the experiment rats were given an overdose of chloral hydrate. The brain was fixed with 4% paraformaldehyde and dialysis probe placement was visually localized according to the atlas of Paxinos & Watson (1986).

Measurement of dopamine

Dopamine level in dialysates was analysed by h.p.l.c. with electrochemical detection. A Merck L-6200 pump was used in conjunction with a glassy carbon electrode set at −780 mV (ANTEC, The Netherlands). A Merck Lichrocart cartridge (125 mm × 4 mm) column filled with Lichrospher reverse-phase C_{18} 5 μM material was used. The mobile phase consisted of a mixture of 0.05 M of sodium acetate, 0.4 mM of 1-octanesulphonic acid, 0.3 mM of Na_2 EDTA and 70 ml methanol l^{-1}, adjusted to pH 4.1 with acetic acid. All reagents and water were h.p.l.c. grade. The flow rate was 0.8 ml min^{-1} and the detection limit for dopamine was 5 fmol per injection.

Measurement of aspartate and glutamate

Aspartate and glutamate levels in dialysates were analysed by h.p.l.c. after precolumn derivatization with *o*-phthalaldehyde (OPA). Dialysates collected at 30 min intervals (90 μl) were diluted to 500 μl with water : metanol 20 : 80. Afterwards, 200 μl of borate buffer (0.5 M, pH 9.5) were added. The mixture was reacted with 100 μl of OPA reagent (100 mg of OPA dissolved in 9 ml of methanol, 1 ml of borate buffer and 100 μl of 2-mercaptoethanol). After 200 s of reaction time, 45 μl of HCl 0.75 N were added to neutralize the reaction mixture. One hundred microlitres of the reaction mixture were mixed with the same volume of the starting eluent and, finally, 20 μl were injected onto the column (Superspher 100 RP 18, 4 μm, Lichrocart 250-4). The precolumn derivatization and injection onto the column were performed automatically by a Merck AS-4000 Autosampler. Separation of amino acids was achieved by use of a gradient elution. Two eluents were used: eluent A (50 mM sodium acetate pH 7.0) and eluent B (methanol). The gradient was held 10 min in 26% methanol, then run from 26 to 38% methanol over 15 min, and from 38 to 75% methanol over another 15 min, and held 3 min in 75% methanol. The effluent was monitored by a fluorescence detector (Merck F-1050) with the excitation and emission wavelengths at 330 and 450 nm, respectively. Aspartate and glutamate were identified and quantified by comparing their retention times and peak areas with those of external standards.

Measurement of ascorbate

Analyses were performed by h.p.l.c. with ultraviolet detection. A Merck L-6200 pump was used in conjuction with an ultraviolet detector (Shimadzu SPD-6AV) set at 254 nm at 0.04 a.u.f.s. Ascorbate was separated with a Merck Lichrocart cartridge column (Licrospher C_{18}, 5 μm, 125 × 4 mm) with 0.05 M sodium acetate and 2% acetonitrile, adjusted to pH 4.3 with acetic acid. All reagents and water were h.p.l.c. grade. The flow rate was 0.5 ml min^{-1}.

Measurement of dopamine uptake

Rat striatal synaptosomes were prepared and incubated in Krebs medium as described previously (Lapchack & Hefti, 1992). Briefly, striatal tissue was homogenized in 0.32 M ice-cold sucrose (10 strokes with a ground glass homogenizer) followed by centrifugation in an Eppendorf microfuge (4500 r.p.m., 10 min) at 4°C. The supernatant was then collected and recentrifuged (14000 r.p.m., 20 min). The pellet was collected and used as the P2 synaptosomal fraction. After resuspension with 100 μl of normal Krebs medium, 20 μl of this solution were preincubated at 37°C for 10 min in 0.5 ml of normal Krebs medium containing ascorbate (1 mg ml^{-1}), pargyline (10 μM), imipramine (10 μM) and increasing concentrations of PDC (10 nM–1 mM). For [^{3}H]-dopamine uptake, 2 μCi of 3,4-[7-^{3}H]-dopamine was added to the solution containing the synaptosomes and incubated for another 5 min. Following the incubation, the synaptosomes were pelleted and solubilized, and the content of [^{3}H]-dopamine was determined by liquid scintillation counting.

Immunohistological evaluation

Tyrosine hydroxylase (TH) immunohistology was carried out 24 h after perfusion of Ringer solution for 3 h, alone or co-perfused with MPP^+ 1 mM for 15 min.

Rats were perfused through the heart under deep anaesthesia (chloral hydrate) with 150–200 ml of 4% paraformaldehyde in phosphate buffer, pH 7.4. The brains were removed, and then cryoprotected serially in sucrose in PBS, pH 7.4; first in 10% sucrose for 24 h and then in 30% sucrose until sunk (2–5 days). The brains were then frozen in isopentane at −15°C, and 25 μm sections were cut on a cryostat and mounted in gelatine-coated slices. All incubations and washes were in Tris buffered saline (TBS), pH 7.4 unless otherwise noted. All work was done at room temperature. Sections were washed and then treated with 0.3% hydrogen peroxide in methanol for 30 min, washed again, and incubated in a solution containing TBS and 1% horse serum for 60 min in a humid chamber. Slices were drained and further incubated with mouse anti-βTH (Boehringer-Mannheim, 1 : 200) in TBS containing 1% horse serum and 0.25% Triton-X-100 for 24 h.

Sections were then incubated for 2 h with biotinylated horse anti-mouse IgG (Vector, 1 : 200) followed by a second 1 h incubation with ExtrAvidin-Peroxidase solution (Sigma, 1 : 100). The antibody was diluted in TBS containing 0.25% Triton-X-100, and its addition was preceded by three 10 min rinses in TBS. The peroxidase was visualized with a standard diaminobenzidine/hydrogen peroxidase chromogen reaction for 5 min.

Drugs

The following drugs were used: MPP^+ iodide (Research Biochemical Inc., Natick, MA, U.S.A.), L-*trans*-pyrrolidine-2,4-dicarboxylic acid (PDC, Tocris Cookson; Bristol, U.K.) and ascorbic acid (AA, Merck; Darmstadt, Germany).

Statistical analysis

Difference between the average dialysate concentrations or the percentage of baseline for the control and drugs treatment was compared by Kruskal-Wallis analysis of variance by ranks, and, where appropriate (H value greater than the 95% confidence level), comparison of the means was carried out with the Mann-Whitney U-test. Unpaired Student's *t* test was used to compare the values of the different treatments at the same collection time.

Results

Effect of perfusion of MPP^+ on tyrosine hydroxylase immunohistology

As the first step, dopaminergic terminal degeneration consequent to the intrastriatal perfusion of MPP^+ was assessed by TH immunoreactivity. Figure 1 shows a tissue section of a representative animal perfused intrastriatally with Ringer solution alone and with the inclusion of MPP^+ 1 mM for 15 min in the Ringer solution. After Ringer solution perfusion alone the general appearance of the tissue was normal, even around the cannula (Figure 1a). In contrast, inclusion of MPP^+ 1 mM for 15 min produced a decrease of TH immunoreactivity around the cannula (Figure 1b).

Basal values

Twenty-four hours after surgery, basal extracellular levels were similar and they were grouped together, being (mean ± s.e.mean): 14.2 ± 0.8 fmol min^{-1} ($n = 47$) for dopamine; 751.0 ± 47.2 fmol min^{-1} ($n = 10$) for glutamate; 187.3 ± 41.4 fmol min^{-1} ($n = 10$) for asparate and 10.7 ± 1.2 pmol min^{-1} ($n = 10$) for ascorbate.

Effect of co-perfusion of PDC with MPP^+ on extracellular output of dopamine

Perfusion of PDC 1 and 10 mM stimulated the basal release of dopamine about 2 and 10 times, respectively (Figure 2). Perfusion of PDC 0.1 mM did not affect the basal release of dopamine (Figure 2). One hour after PDC perfusion started, MPP^+ 1 mM was co-perfused with PDC for 15 min. The increase in the extracellular output of dopamine produced by MPP^+ perfusion was inversely proportional to the concentration of PDC previously perfused (Figure 3a). Twenty four hours later, the possible neuroprotective effect of PDC against MPP^+ toxicity was assessed by the increase in the extracellular output of dopamine produced by a second perfusion of MPP^+ 1 mM for 15 min. The amount of dopamine measured after this second MPP^+ perfusion should be proportional to the number of dopaminergic terminals remaining after the first MPP^+ perfusion. PDC 1 mM perfusion produced the smallest increase in the extracellular output of dopamine, followed by control perfusion, while the highest increase was produced by PDC 10 mM perfusion (Figure 3b).

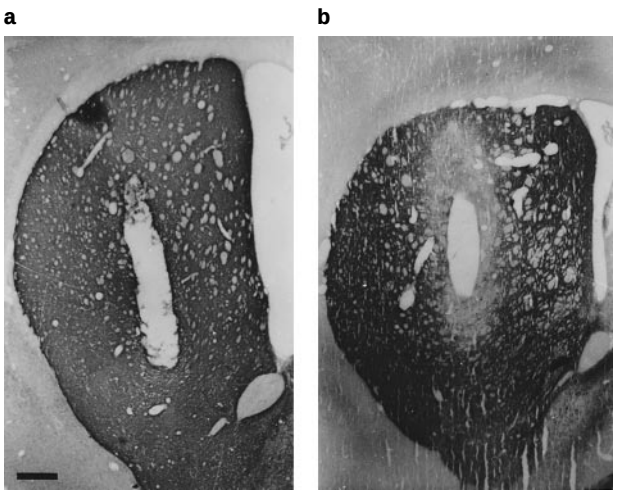

Figure 1 Photomicrograph of coronal sections (cryostat-cut sections) through striatum after tyrosine hydroxylase (TH) immunostaining. (a) Striatum of a rat perfused with Ringer solution. Note the normal appearance of TH immunoreactivity terminals around the cannula. (b) Striatum of a rat perfused with MPP^+ 1 mM for 15 min. In contrast, there is a specific absence of TH immunoreactivity around the cannula, demonstrating the extent of loss of TH. The magnification bar represents 500 μm.

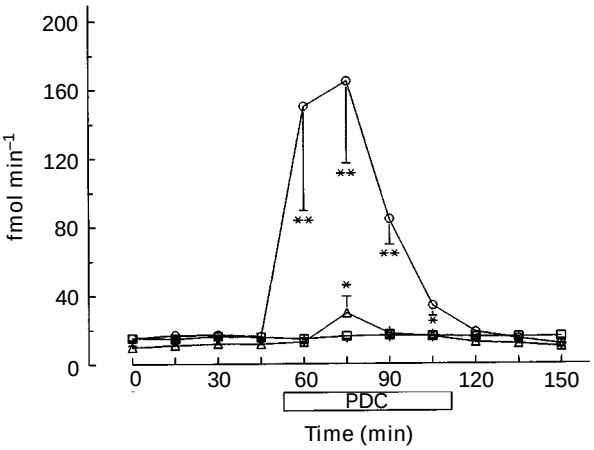

Figure 2 Effect of perfusion of PDC (□) 0.1, (△) 1 and (○) 10 mM on the striatal extracellular output of dopamine. Data are mean ± s.e.mean (vertical lines), expressed as fmol min^{-1} ($n = 5$). Statistical significance (Kruskal-Wallis followed by Mann-Whitney's U-test): $^*P < 0.05$; $^{**}P < 0.01$, compared with the control value.

Effect of PDC on dopamine uptake

Five different concentrations of PDC were tested in striatal synaptosomes. Control (without PDC) and PDC 0.1, 1, 10 and 100 μM uptake data, in pmol dopamine μg^{-1} h^{-1} protein (mean ± s.d., $n = 5$), were 3.63 ± 0.15, 3.46 ± 0.13, 3.57 ± 0.22, 3.66 ± 0.33, and 3.61 ± 0.23, respectively. Only the highest concentration of PDC studied (1000 μM) produced a significant increase in the uptake of dopamine: 4.28 ± 0.39 ($n = 5$; $P < 0.01$, compared with control data, Student's *t* test).

Effect of co-perfusion of PDC with MPP^+ on the extracellular output of glutamate or aspartate

Perfusion of MPP^+ 1 mM for 15 min produced a small but statistically significant increase in the extracellular output of glutamate or aspartate (Figure 4a). Perfusion of PDC 10 mM stimulated the extracellular output of glutamate or aspartate (Figure 4a). Its co-perfusion with MPP^+ 1 mM did not pro-

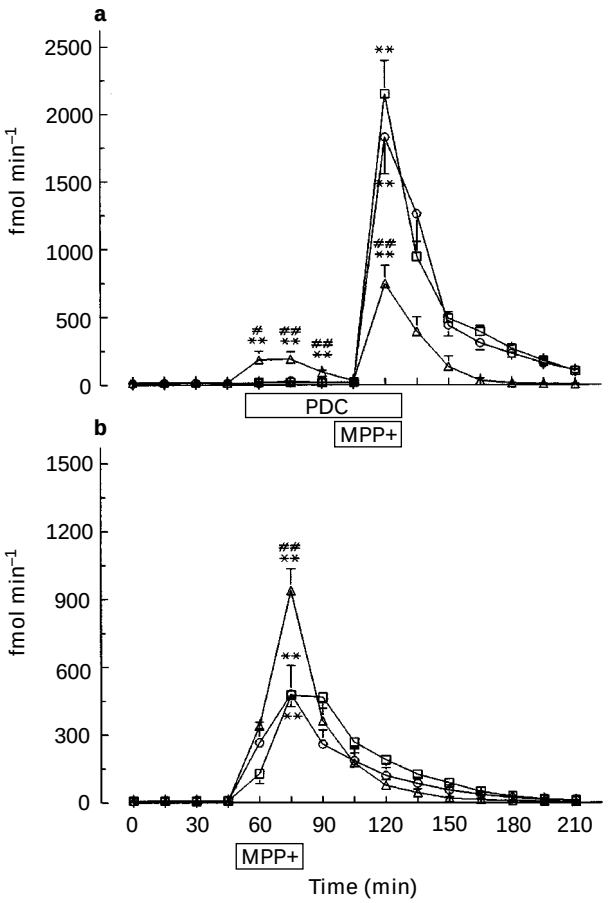

Figure 3 (a) Effect of perfusion of MPP^+ 1 mM alone (□) or with PDC 1 (○) and 10 (△) mM on the striatal extracellular output of dopamine. (b) Effect of perfusion of MPP^+ 1 mM, 24 h after perfusion of MPP^+ 1 mM alone or with PDC 1 and 10 mM, on the striatal extracellular output of dopamine. Data are mean ± s.e.mean (vertical lines), expressed as fmol min^{-1} ($n = 5-7$). Statistical significance (Kruskal-Wallis followed by Mann-Whitney's U-test): $^{**}P<0.01$, compared with the control value. $^{\#}P<0.05$, $^{\#\#}P<0.01$, compared with perfusion of MPP^+ alone at the same collection time (Student's t test).

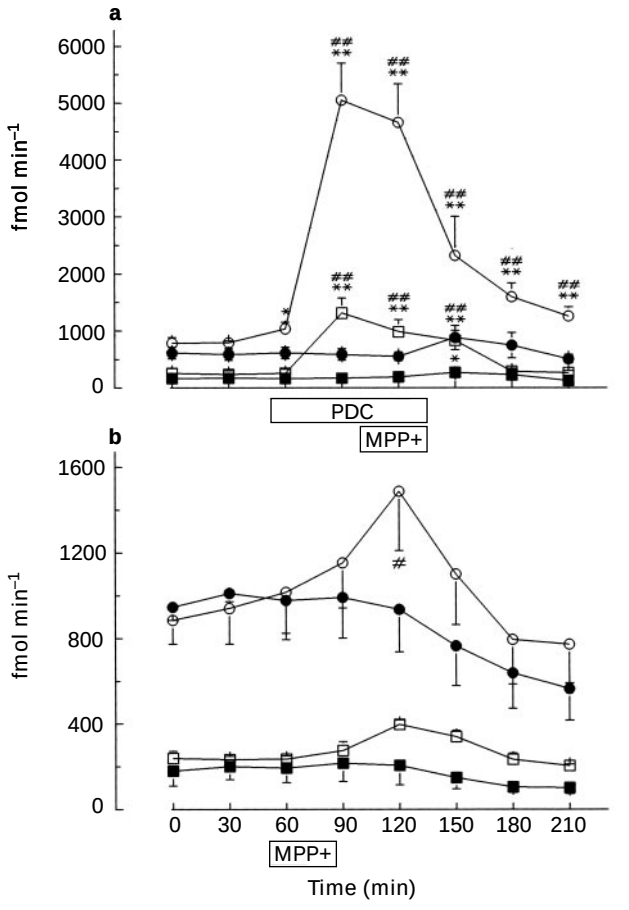

Figure 4 (a) Effect of perfusion of MPP^+ 1 mM alone (●, ■) or with PDC 10 mM (○, □) on the striatal extracellular output of (●, ○) glutamate or (■, □) aspartate. (b) Effect of perfusion of MPP^+ 1 mM, 24 h after perfusion of MPP^+ 1 mM alone or with PDC 10 mM, on the striatal extracellular output of glutamate or aspartate. Data are mean ± s.e.mean (vertical lines), expressed as fmol min^{-1} ($n = 5$). Statistical significance (Kruskal-Wallis followed by Mann-Whitney's U-test): $^{*}P<0.05$; $^{**}P<0.01$, compared with the control value. $^{\#\#}P<0.01$, compared with perfusion of MPP^+ alone at the same collection time (Student's t test).

duce any further increase in the extracellular output of glutamate or aspartate. Twenty four hours later, a second perfusion of MPP^+ 1 mM for 15 min did not produce any significant increase in the extracellular output of glutamate or aspartate, with the exception of the experiment in which PDC 10 mM and MPP^+ 1 mM were perfused on the first day (Figure 4b). Aspartate overflow increased after the second MPP^+ perfusion.

Effect of co-perfusion of PDC with MPP^+ on the extracellular output of ascorbate

Perfusion of PDC 10 mM produced a statistically significant decrease in the extracellular output of ascorbate on the first day (Figure 5a). MPP^+ 1 mM alone or co-perfused with PDC 10 mM did not produce any significant effect on the extracellular output of ascorbate on the first day (Figure 5a). A second perfusion of MPP^+ 1 mM for 15 min one day later produced a significant increase and decrease when it had been perfused, respectively, without and with PDC 10 mM the day before (Figure 5b).

Effect of co-perfusion of PDC with ascorbate and MPP^+ on the extracellular output of dopamine

The protective effect observed with PDC could be due to its ability to decrease extracellular ascorbate concentration. Perfusion of four different concentrations of ascorbate (0.05, 0.5, 2 and 10 mM) stimulated the basal release of dopamine. Their co-perfusion with MPP^+ 1 mM for 15 min on the first day did not affect MPP^+ toxicity on the second day. The increase in the extracellular output of dopamine after MPP^+ perfusion on the first day was always about 1200 fmol min^{-1} (data not shown). Therefore, due to the similarity of results observed with every ascorbate concentration, we decided to use a single concentration of 0.5 mM ascorbate for its co-perfusion with PDC.

Inclusion of ascorbate 0.5 mM for 1 h before MPP^+ 1 mM perfusion produced increases in the extracellular output of dopamine similar to or higher than those produced by PDC 10 mM (Figure 6a). Co-perfusion of PDC with ascorbate reduced the increase in the extracellular output of dopamine produced by MPP^+ 1 mM perfusion (Figure 6a). The dopamine extracellular output increase after the second MPP^+ perfusion was similar whether ascorbate was absent or present in the Ringer solution for 1 h before the first MPP^+ perfusion (Figure 6b). Co-perfusion of ascorbate with PDC completely prevented the neuroprotective effect against MPP^+ toxicity (Figure 6b).

Table 1 shows the maximum extracellular output of dopamine after MPP^+ 1 mM perfusion, 48 h after surgery, and presents a summary of the effect of the different treatments carried out 24 h after surgery.

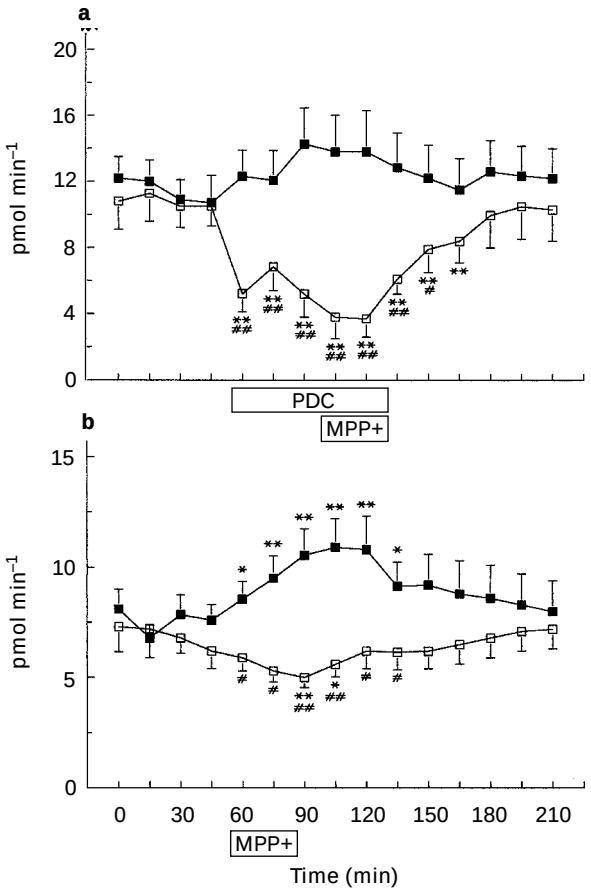

Figure 5 (a) Effect of perfusion of MPP^+ 1 mM alone (■) or with PDC 10 mM (□) on the striatal extracellular output of ascorbate. (b) Effect of perfusion of MPP^+ 1 mM, 24 h after perfusion of MPP^+ 1 mM alone or with PDC 10 mM, on the striatal extracellular output of ascorbate. Data are mean ± s.e.mean (vertical lines), expressed as pmol min^{-1} ($n=5$). Statistical significance (Kruskal-Wallis followed by Mann-Whitney's U-test): $^*P<0.05$; $^{**}P<0.01$, compared with the control value. $^{\#}P<0.05$; $^{\#\#}P<0.01$, compared with perfusion of MPP^+ alone at the same collection time (Student's t test).

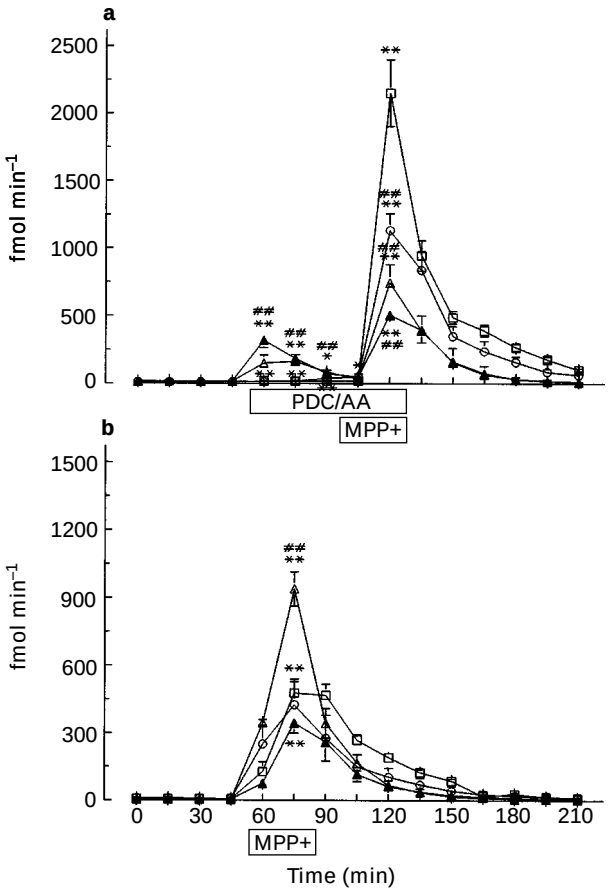

Figure 6 (a) Effect of perfusion of MPP^+ 1 mM alone (□), with PDC 10 mM (△), with ascorbate 0.5 mM (○) or with PDC 10 mM plus ascorbate 0.5 mM (▲) on the striatal extracellular output of dopamine. (b) Effect of perfusion of MPP^+ 1 mM, 24 h after perfusion of MPP^+ 1 mM alone, with PDC 10 mM, with ascorbate 0.5 mM or with PDC 10 mM plus ascorbate 0.5 mM, on the striatal extracellular output of dopamine. Data are mean ± s.e.mean (vertical lines), expressed as fmol min^{-1} ($n=5-7$). Statistical significance (Kruskal-Wallis followed by Mann-Whitney's U-test): $^*P<0.05$; $^{**}P<0.01$, compared with the control value. $^{\#\#}P<0.01$, compared with perfusion of MPP^+ alone at the same collection time (Student's t test).

Discussion

It has been suggested that glutamate-induced excitotoxicity could be involved in the neurotoxicity produced by MPTP/MPP^+ (Olney, 1989), which is consistent with the protective effect described for different NMDA receptor antagonists (Turski *et al.*, 1991; Zuddas *et al.*, 1992; Srivastasva *et al.*, 1993; Chan *et al.*, 1993). Thus, it would be expected that an increase in extracellular glutamate concentration, following an inhibition of glutamate uptake, would result in a potentiation of the damage to dopaminergic terminals produced by MPP^+ perfusion. Our results confirm previous data showing a significant increase in glutamate and aspartate extracellular concentrations after perfusion of L-*trans*-pyrrolidine-2,4-dicarboxylic acid (PDC) (Massieu *et al.*, 1995). However, an unexpected result was found: perfusion of the glutamate uptake inhibitor PDC protected dopaminergic terminals from MPP^+ toxicity. This result seems to demonstrate that protection is due to PDC inhibition of glutamate uptake, through the glutamate/ascorbate heteroexchange system (Miele *et al.*, 1994). It is important to point out that, in contrast with other authors (Westerink *et al.*, 1987; Carboni *et al.*, 1990), we did not find any increase in glutamate/aspartate extracellular output after MPP^+ perfusion. We assume that such an increase is masked by the high activity of the glutamate/aspartate uptake system. Since changes in glutamate/aspartate levels after MPP^+ co-perfusion with PDC were not observed, MPP^+, at the doses used in our experiments, appears to destroy selectively dopaminergic, but not glutamatergic, nerve terminals.

Table 1 Effect of perfusion of MPP^+ 1 mM, 24 h after a previous MPP^+ perfusion, on the striatal extracellular output of dopamine

Treatment on day 1	*Maximum output of dopamine*
MPP^+ 1 mM alone	476.8 ± 49.6
+ PDC 1 mM	480.9 ± 126.7
+ PDC 10 mM	939.4 ± 75.1**
+ AA 0.5 mM	425.1 ± 113.4
+ PDC 10 mM + AA 0.5 mM	344.2 ± 45.7

Data are means ± s.e.mean values ($n=5$) expressed as fmol/min. Statistical significance (Student's t-test): $^{**}P<0.01$ compared with perfusion of MPP^+ alone. (See Materials and methods for perfusion times.)

Our results are in line with those demonstrating no protection against MPP^+ toxicity with the glutamate receptor antagonist, dizolcipine (MK-801) (Sonsalla *et al.*, 1989; 1992; Kupsch *et al.*, 1992; Finiels-Marie *et al.*, 1993) and with others

who demonstrated protection against different types of neuronal damage despite an increased extracellular glutamate. For example, Massieu *et al*. (1995) showed that accumulation of extracellular glutamate is not sufficient for inducing neuronal damage. Similarly, Dijk *et al*. (1994) found that 24 h food deprivation protects rat striatum against hypoxic-ischaemic damage despite high extracellular glutamate, and glutamate rises to the same extent in the CA_1 and CA_3 areas of the hippocampus, although the resistance of the two areas to ischaemia is quite different (Mitani *et al*., 1992).

Assuming that the protection of striatal dopaminergic neurones observed in this study is due to the inhibition of the glutamate/aspartate uptake, through the glutamate/ascorbate heteroexchange system, several explanations could account for this effect. One likely explanation is the decrease in MPP^+-induced dopamine release the first day in rats perfused with PDC one hour before MPP^+ perfusion. However, it is interesting to note that PDC perfusion alone produced a significant increase in dopamine release. These results are in agreement with others which have shown that glutamate or its agonists evoke dopamine release *in vitro* or *in vivo* (Clow & Jhamandas, 1989; Leviel *et al*., 1990; Carrozza *et al*., 1991). However, PDC perfusion led to a significant decrease of dopamine overflow produced by MPP^+ perfusion. Dopamine overflow appears to be a critical link in the chain of events underlying neurotoxicity because manipulations designed to reduce dopaminergic transmission (dopamine antagonists, synthesis inhibitors and nigrostriatal tract lesion) all block methamphetamine and methylenedioxy-methamphetamine toxicity (Gibb *et al*., 1990).

As has been shown for MK-801 (Clarke & Reuben, 1995; Venero *et al*., 1996) another possible mechanism by which PDC could prevent the striatal depletion produced by MPP^+ is through inhibition of the dopamine transporter. Inhibition of dopamine uptake protects against MPTP/MPP^+ (Martin *et al*., 1991) and methamphetamine toxicity (Marek *et al*., 1990). In contrast with MK-801, PDC did not show an inhibitory effect on the uptake of dopamine.

Our results show that perfusion of PDC produced an increase in extracellular glutamate and aspartate and, at the same time, a decrease in extracellular ascorbate, consistent with the functioning of a glutamate/ascorbate heteroexchange system (Cammack *et al*., 1991; Grunewald, 1993; Miele *et al*., 1994; Walker *et al*., 1995). Intraneostriatal infusion of the competitive N-methyl-D-aspartate (NMDA) antagonist 3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonene (CPPene; 5 $\mu g\ \mu l^{-1}$) or non-competitive NMDA antagonist MK-801 (3 $\mu g\ \mu l^{-1}$) decreased neostriatal ascorbate release, probably by decreasing extracellular glutamate, since glutamate perfusion produced an increase in the release of ascorbate in the presence of MK-801 (Pierce & Rebec, 1992; 1993). These results led us to consider a third possibility to explain the protective action of glutamate uptake inhibition against toxic damage: the ascorbate hypothesis. While PDC decreased extracellular ascorbate levels, it was not effective after MPP^+ perfusion. If this decrease in ascorbate output was the cause of the protective effect of PDC it should be reversed after coperfusion of PDC with ascorbate. Perfusion of PDC plus ascorbate completely abolished the protective effect of PDC, suggesting that ascorbate is involved in MPP^+ neurotoxicity. However, perfusion of different concentrations of ascorbate for 1 h before MPP^+ perfusion did not produce an increase in MPP^+ toxicity. Therefore, a likely explanation for this effect is that the neuroprotective action of PDC is due to a decrease in extracellular ascorbate levels. Our results indicate that MPP^+ toxicity is not due to an effect on the amount of dopamine released after the first MPP^+ perfusion, since ascorbate plus PDC reinstated the damage produced by MPP^+, whereas ascorbate plus PDC released even less dopamine after MPP^+ perfusion than PDC alone on the first day. These results are in line with a recent study which showed that MPP^+ toxicity is lower in striatal slices from guinea-pigs fed with a diet lacking vitamin C (Revuelta *et al*., 1997). Similar results were obtained *in vivo* by Perry *et al*. (1985). The involvement of extracellular ascorbate in MPP^+-induced toxicity could be related to its anti-oxidant/pro-oxidant activity (Halliwell, 1996). In the presence of high iron levels ascorbate can act as a pro-oxidant, helping to generate iron-catalysed peroxides that can destroy dopaminergic neurones (Kehrer, 1993; Li *et al*., 1995). The involvement of iron in MPP^+ toxicity has been recently described (Santiago *et al*., 1997). An increase in iron extracellular concentration after MPP^+ perfusion, along with the presence of ascorbate in the extracellular space, could increase the oxidative damage.

In conclusion, our results suggest that high glutamate/aspartate levels do not increase MPP^+ toxicity; protective effect was even observed when a high concentration (10 mM) of the inhibitor of glutamate uptake was perfused. Therefore glutamate-induced excitotoxicity is unlikely to be involved in the mechanisms underlying MPP^+ toxicity. The protective effect found with PDC 10 mM could be due to a decrease in extracellular ascorbate levels, suggesting that ascorbate is involved in the oxidative stress induced by MPP^+.

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