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Manganese increases L-DOPA auto-oxidation in the striatum of the freely moving rat: potential implications to L-DOPA long-term therapy of Parkinson's disease

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1 We have previously shown that manganese enhances L-dihydroxyphenylanine (L-DOPA) toxicity to PC12 cells *in vitro*. The supposed mechanism of manganese enhancing effect [an increase in L-DOPA and dopamine (DA) auto-oxidation] was studied using microdialysis in the striatum of freely moving rats.

2 Systemic L-DOPA [25 mg kg^{-1} intraperitoneally (i.p.) twice in a 12 h interval] significantly increased baseline dialysate concentrations of L-DOPA, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and uric acid, compared to controls. Conversely, DA and ascorbic acid concentrations were significantly decreased.

3 A L-DOPA oxidation product, presumptively identified as L-DOPA semiquinone, was detected in the dialysate. The L-DOPA semiquinone was detected also following intrastriatal infusion of L-DOPA.

4 In rats given L-DOPA i.p., intrastriatal infusion of N-acetylcysteine (NAC) significantly increased DA and L-DOPA dialysate concentrations and lowered those of L-DOPA semiquinone; in addition, NAC decreased DOPAC + HVA and uric acid dialysate concentrations.

5 In rats given L-DOPA either systemically or intrastriatally, intrastriatal infusion of manganese decreased L-DOPA dialysate concentrations and greatly increased those of L-DOPA semiquinone. These changes were inhibited by NAC infusion.

6 These findings demonstrate that auto-oxidation of exogenous L-DOPA occurs *in vivo* in the rat striatum. The consequent reactive oxygen species generation may account for the decrease in dialysate DA and ascorbic acid concentrations and increase in enzymatic oxidation of xanthine and DA. L-DOPA auto-oxidation is inhibited by NAC and enhanced by manganese. These results may be of relevance to the L-DOPA long-term therapy of Parkinson's disease.
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Keywords: L-DOPA; L-DOPA semiquinone; dopamine; *in vivo* auto-oxidation; manganese; microdialysis; rat striatum

Abbreviations: ANOVA, analysis of variance; DA, dopamine; DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid; i.p., intraperitoneally; L-DOPA, L-dihydroxyphenylanine; NAC, N-acetylcysteine; ROS, reactive oxygen species; XO, xanthine oxidase

Introduction

Parkinson's disease is characterized by selective loss of neurons in the *substantia nigra pars compacta* and significant reduction of neostriatal content of dopamine (DA) and its major acidic metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA); consequently, the functioning of the nigro-striatal dopaminergic system is impaired. Parkinsonian symptoms are relieved by administration of L-dihydroxyphenylalanine (L-DOPA), which is converted by neuronal aromatic L-amino acid decarboxylase (EC 4.1.1.28) into DA, hence restoring DA levels in surviving neurons, which, however, continue to die despite the L-DOPA treatment (Basma *et al*., 1995). Oxidative stress is widely believed an important pathogenetic mechanism of neuronal death in Parkinson's disease (Halliwell, 1992), although it is still not clear whether it is an initial event causing cell death or a consequence of the disease (Jellinger, 1999). L-DOPA can undergo auto-oxidation (Parsons, 1985) to generate quinones and reactive oxygen species (ROS), which would further load the pre-existing condition of oxidative stress at nigro-striatal sites (Basma *et al*., 1995; Spencer *et al*., 1995). Smith *et al*. (1994) have shown that L-DOPA increases nigral hydroxyl radical production in the freely moving rat. The quinones generated by L-DOPA oxidation react with cysteine to form 5-S-cysteinyl-DOPA. Spencer *et al*. (1998) have shown that cysteinyl-conjugates of catecholamine in Parkinson's disease are higher than in normal *substantia nigra*. DA and L-DOPA have been shown to induce oxidative stress-mediated apoptosis in cultured neuronal cells, and inappropriate DA- and/or L-DOPA-induced activation of apoptosis might have a role in neuronal death in Parkinson's disease (Ziv *et al*., 1994; 1997; Walkinshaw & Waters, 1995; Hastings *et al*., 1996). Death of nigral dopaminergic neurons in Parkinson's disease has been reported to occur by apoptosis (Mochizuki *et al*., 1996); according to Anglade *et al*. (1997), even at the final state of the disease the dopaminergic neurons undergo active process of cell death.

Chronic manganese intoxication in man can induce a Parkinson-like syndrome (Donaldson, 1987). Manganese may stimulate DA auto-oxidation within the dopaminergic neuron,

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a process accompanied by an increase in formation of quinones (Florence & Stauber, 1989; Shen & Dryhurst, 1998) and protein-bound cysteinyl DA and cysteinyl DOPAC (Hastings *et al*., 1996). We have shown that manganese induces oxidative stress-mediated apoptosis in PC12 cells, a catecholamine-containing cell line (Desole *et al*., 1997). In addition, we have shown (Migheli *et al*., 1999) that concentrations of L-DOPA non-toxic to PC12 cells, when associated with a sub-toxic manganese concentration (i.e. unable to induce impairment of dopamine metabolism, decrease in cell viability and apoptosis), become highly toxic. The antioxidants N-acetylcysteine (NAC) and (to a lesser extent) ascorbic acid (AA) protected from L-DOPA + manganese cytotoxicity. An increase in L-DOPA and DA auto-oxidation has been suggested as mechanism of manganese enhanced toxicity (Migheli *et al*., 1999). The present microdialysis study has been performed in the striatum of freely moving rats in order to assess whether our *in vitro* findings on the manganese and L-DOPA interaction could be extended to the *in vivo* situation. The results demonstrate that: (i) auto-oxidation of exogenous L-DOPA occurs *in vivo* in the rat striatum; (ii) the auto-oxidation is greatly increased by manganese, and (iii) is inhibited by NAC.

Methods

Animals

Male Wistar rats (Morini, R. Emilia, Italy), weighing between 280–330 g were used in all experiments. The rats were maintained under standard animal care conditions (12:12 h light/dark cycle, lights coming on at 07.00 h; room temperature 21°C), with food and water *ad libitum*. Prior to the start of any experiment, the health of the rat was assessed according to published guidelines (Morton & Griffiths, 1985). All procedures were specifically licensed under the European Community directive 86/609 included in Decreto No. 116/1992 of the Italian Ministry of Public Health.

Drugs

L-DOPA, NAC, ascorbic acid, and 2,4,5-trihydroxyphenylalanine were purchased from Sigma-Aldrich (Milan, Italy); manganese chloride ($MnCl_2$) and d-amphetamine sulphate from Merck (Darmstadt, Germany); L-DOPA 3-4-orthoquinone and its cyclization derivative o-quinone imine (Shen & Dryhurst, 1998) were synthesized by one of us (G. Grella).

Drug administration

The $MnCl_2$ concentration (30 mM) infused intrastriatally and the dose of systemic L-DOPA [25 mg kg^{-1} intraperitoneally (i.p.) twice in a 12 h interval] were chosen according to Parenti *et al*. (1986); NAC concentration (0.1 mM) was chosen according to the previous study (Migheli *et al*., 1999).

Microdialysis probe construction

The striatal probe combined two independent microdialysis probes of concentric design with two separate inlets and a shared outlet, as previously described (Miele *et al*., 2000). The probes were constructed using two sections of plastic-coated silica tubing (diameter 0.15 mm; Scientific Glass Engineering, Milton Keynes, U.K.) each placed in the centre of a semi-permeable polyacrylonitrile dialysis fibres (molecular cut-off weight of 12 KD, Filtral 16 Hospal Industrie, France). Each probe had a final diameter of 0.22 mm. The tips of the dialysis fibres were sealed and joined using quick-drying epoxy glue. The two silica tubing served as inlets; the outlet was made also with a section of plastic-coated silica tubing, positioned in the centre of polythene tubing. The semi-permeable membrane was coated with epoxy leaving an active length of 4 mm. The diameter of the final probe was approximately 0.50 mm. The striatal probe combining two microdialysis probes of concentric design with two separate inlets and a shared outlet, allowed us to infuse $MnCl_2$ during continuous infusion of either NAC or L-DOPA. This procedure also prevented manganese-induced auto-oxidation of L-DOPA in the probe.

Surgery

Stereotaxic surgery was performed under chloral hydrate (400 mg kg^{-1} i.p.) anaesthesia. The microdialysis probes were implanted in the right striatum using the following co-ordinates from the atlas of Paxinos & Watson (1986): A/P +0.5 from bregma, +2.5 M/L, and −6.0 D/V from dura. Body temperature during anaesthesia was maintained at 37°C by means of an isothermal-heating pad. Following surgery the animals were placed in large plastic bowls (50 × 55 cm), and maintained in a temperature- and light-controlled environment, with free access to food and water. Experiments were carried out 24 h after probe implantation with the animal in its home bowl. This arrangement allowed the rats free movement.

Microdialysis procedure

The composition of the Ringer solution used was as follows (in mM): NaCl 147, KCl 4, $CaCl_2$ 1.2, $MgCl_2$ 1. A microinfusion pump (CMA/100, Microdialysis, Sweden) pumped Ringer solution at a flow rate of 1.5 μl min^{-1} using two separate syringes connected to the inlets by a length of polythene tubing; every 20 min, 60 μl dialysate samples were collected manually in 250 μl micro-centrifuge tubes (Alpha Laboratories, U.K.) attached to the outlet. Subsequently, a 20 μl aliquot of collected dialysate was injected into the analytical system. Drugs were added to the Ringer solution and infused *via* the striatal probe implanted in the striatum.

Chromatographic analysis

L-DOPA, L-DOPA semiquinone, DA, DOPAC, HVA, ascorbic acid and uric acid were quantified by high performance liquid chromatography with electrochemical detection (HPLC-EC) as previously described (Enrico *et al*., 1997; Miele *et al*., 2000), using an Alltech 426 HPLC pump equipped with a Rheodyne injector, column 15 cm × 4.6 mm i.d. Alltech Adsorbsphere C18 5U, electrochemical detector Antec CU-04-AZ and Varian Star Chromatographic Workstation. The mobile phase was citric acid 0.5 M, Na acetate 1 M, EDTA 12.5 mM, MeOH 10% and sodium octylsulphate 650 mg l^{-1} (pH = 3.0); the flow rate was 1.3 ml min^{-1}. The first sample was collected after 60 min of stabilization (time 0), then dialysates were collected, at 20 min intervals, for 40 min prior to the start of experiments.

Histology

Following the experiments, rats were killed with an overdose of chloral hydrate (800 mg kg^{-1}, i.p.). The location of each microdialysis probe was confirmed by post-mortem histology. Brains were fixed in formal saline and 50 μm coronal sections

were made with a cryostat. The slices were stained with cresyl violet and examined under a microscope.

Statistical analysis

The concentrations in the dialysate were expressed in nM (DA, L-DOPA, L-DOPA semiquinone) or μM (DOPAC, HVA, ascorbic acid and uric acid) and given as mean $\pm$ s.e.mean. Drug effects on neurochemicals were statistically evaluated in terms of changes in absolute dialysate concentrations. In some instances, changes were also evaluated as percentage of baseline. Statistical significance was assessed using analysis of variance (ANOVA) for difference between groups and over time. Difference within or between groups were determined by paired or unpaired *t*-tests with Bonferroni multiple comparison adjustment. Pearson's correlation coefficient between individual concentrations of L-DOPA and L-DOPA semiquinone was calculated in some instances.

Results

Detection of a L-DOPA auto-oxidation product in vivo *and* in vitro

Following L-DOPA 1 μM intrastriatal infusion, HPLC-EC chromatograms of the striatal dialysate revealed a peak (retention time 5.9 min) which was not present in the striatal dialysate of untreated rats. The peak appeared within 20 min from the start of L-DOPA infusion. The peak area progressively increased during continuous L-DOPA infusion (see below). The same peak was detected in an aqueous solution of L-DOPA (1 μM) and $MnCl_2$ (1 μM) after alkalinization (Klegeris *et al*., 1995) (Figure 1). The peak appeared within 5 min and progressively increased up to 5 h, then declined. In contrast, the L-DOPA peak progressively decreased: the amount of L-DOPA oxidized was about 60% after 5 h, and it increased up to 85% after 8 h. Both NAC (1 μM) and AA (1 μM) strongly inhibited L-DOPA oxidation and the consequent L-DOPA oxidation product formation.

The peak was identified tentatively as L-DOPA semiquinone on the following basis: (a) the non-enzymatic oxidation of L-DOPA gives rise to an o-semiquinone which, after disproportionation, gives rise to the corresponding o-quinone (Spencer *et al*., 1998). L-DOPA, 3,4-orthoquinone could be detected neither after its direct injection in the HPLC apparatus, in the dialysate following its direct injection in the striatum, nor in the aqueous solution of L-DOPA (1 μM) + $MnCl_2$ (1 μM) after alkalinization, since pertinent HPLC-EC chromatograms did not reveal peaks. Obviously, this doesn't rule out the formation of L-DOPA 3,4-orthoquinone from the corresponding semiquinone; it simply means that our HPLC apparatus was not suitable for the detection of the former; (b) the L-DOPA 3,4-orthoquinone cyclization derivative (o-quinone imine) (Shen & Dryhurst, 1998) could be detected neither after its direct injection in the HPLC apparatus, nor in the dialysate following its direct injection in the striatum, since the pertinent HPLC-EC chromatograms did not reveal peaks; (c) the peaks of L-DOPA oxidation product and 2,4,5-trihydroxyphenylalanine had different retention times; (d) the formation of L-DOPA oxidation product occurs *in vitro* within a few minutes, a time consistent with L-DOPA semiquinone formation. In addition, it has been shown that a DA semiquinone free radical is formed *in vitro* following manganese-catalyzed auto-oxidation of dopamine (Florence & Stauber, 1989).

L-DOPA semiquinone could not be synthesized, owing to its charged chemical structure. Therefore, a rough calculation of its concentrations in dialysate samples was made on the basis of the amount of L-DOPA oxidized *in vitro* related to the peak area of the oxidation product. As shown in Figure 2, L-DOPA semiquinone detection occurred about 20 min after the start of L-DOPA (1 μM) intrastriatal infusion. Dialysate concentrations of L-DOPA semiquinone increased over-time, while those of L-DOPA decreased, despite the continuous infusion of L-DOPA. Individual dialysate concentrations of L-DOPA were negatively correlated with those of L-DOPA semiquinone [r values range between -0.767 ($P<0.005$) and -0.831 ($P<0.0001$), df = 10].

Effects of systemic L-DOPA on striatal baseline dialysate concentrations of neurochemicals

The effect of systemic administration of L-DOPA (25 mg kg^{-1} i.p. twice at 12 h intervals) was evaluated by comparing baseline striatal dialysate concentrations of neurochemicals (L-DOPA, DA, DOPAC, HVA, ascorbic acid and uric acid) with

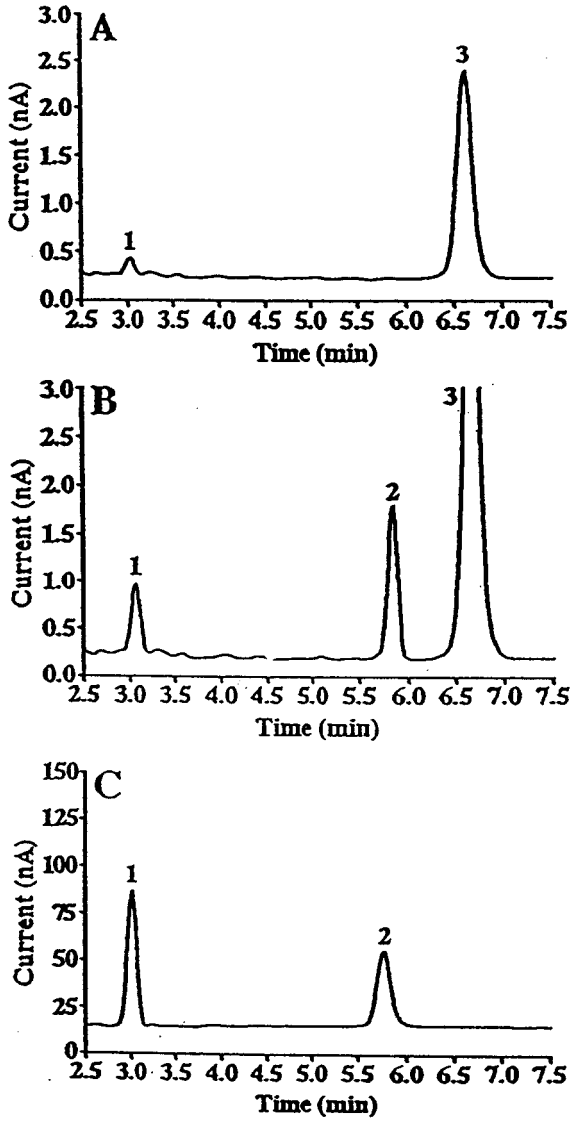

Figure 1 HPLC-EC chromatograms of striatal dialysate from a control rat (A), striatal dialysate from a rat given systemic L-DOPA (B) and an *in vitro* aqueous solution of L-DOPA (1 μM) and $MnCl_2$ (1 μM) after alkalinization (C). 1, L-DOPA peak; 2, L-DOPA semiquinone peak, 3, DOPAC peak.

those detected in controls (unpaired *t*-test). Baseline concentrations were determined 1 h after last L-DOPA administration. The results are given in Table 1. L-DOPA treatment significantly increased baseline dialysate concentrations of L-DOPA (+631.4%), DOPAC (+63.6%), HVA (+93.7%) and uric acid (+82.9%), while it decreased those of DA (−65.2%) and ascorbic acid (−49.9%). These data show that systemic L-DOPA increased both xanthine and DA oxidative metabolism; in addition, systemic L-DOPA did not interfere with DOPAC enzymatic O-methylation to HVA. The decrease in DA dialysate concentration appears to be the consequence of an increased rate of DA auto-oxidation in the dialysate rather than a decrease in neuronal content of DA. In fact, a challenge intrastriatal infusion of d-amphetamine (0.2 mM), at the end of each experiment, increased dialysate DA to up 1500% of preceding concentration both in controls and in L-DOPA-pretreated rats.

The calculated baseline dialysate concentration of L-DOPA semiquinone in rats given systemic L-DOPA was 45.10±2.89 nM ($n=16$) (Table 1). As shown in Figure 2 and Table 1, L-DOPA semiquinone could not be detected in controls. In four rats given systemic L-DOPA alone (25 mg kg^{-1} i.p. twice), the individuals dialysate concentrations of L-DOPA (Figure 3A) were negatively correlated with those of L-DOPA semiquinone (Figure 3B) [r values range between −0.695 ($P<0.01$) and −0.905 ($P<0.0001$), df=12].

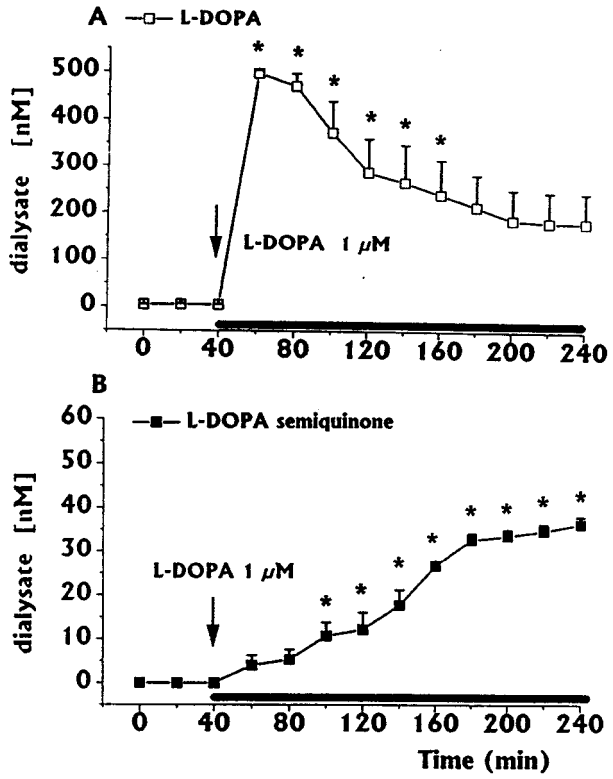

Figure 2 Detection of L-DOPA semiquinone (B) following intrastriatal infusion of L-DOPA. Dialysates were collected, at 20 min intervals, for 200 min during continuous intrastriatal infusion of L-DOPA 1 μM (horizontal black bar). Values are given as mean± s.e.mean ($n=3$). Baseline dialysate concentration of L-DOPA (A) was 4.7±0.76 nM. L-DOPA semiquinone was detected 20 min after the start of L-DOPA infusion. *$P<0.05$ compared with baseline values.

Table 1 Effects of systemic L-DOPA on baseline striatal dialysate concentration of neurochemicals in freely moving rats

	Treatment	
Neurochemical	*None* (n=11)	*L-DOPA* (n=16)
DA (nM)	3.28±0.15	1.15±0.29* (−65.2%)
DOPA (nM)	3.25±0.42	20.52±1.28* (+631.4%)
L-DOPA semiquinone (nM)	ND	45.10±2.89
DOPAC (μM)	0.99±0.07	1.62±0.08* (+63.6%)
HVA (μM)	0.63±0.07	1.22±0.09* (+93.7%)
Uric acid (μM)	1.76±0.10	3.22±0.16* (+82.9%)
Ascorib acid (μM)	10.11±0.74	5.07±0.31* (−49.9%)

L-DOPA 25 mg kg^{-1} was given twice i.p. at 12 h interval. Baseline dialysate concentrations of striatal neurochemicals were determined 1 h after last L-DOPA administration. ND=not detectable. Data are given as mean±s.e.mean. *Statistically different from control ($P<0.001$, unpaired *t*-test).

Effect of intrastriatal NAC infusion of L-DOPA auto-oxidation

In four rats given systemic L-DOPA (25 mg kg^{-1} i.p. twice), the continuous intrastriatal infusion of NAC (0.1 mM for

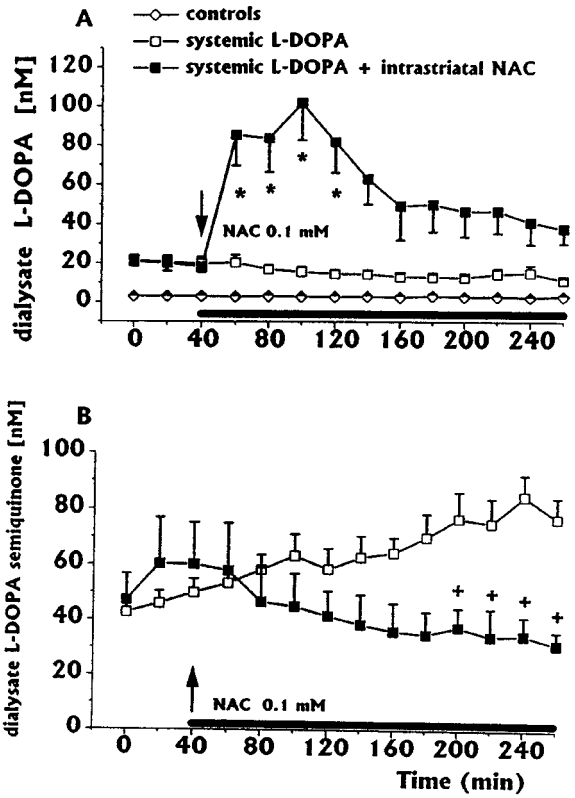

Figure 3 Effect of intrastriatal infusion of NAC on L-DOPA (A) and L-DOPA semiquinone (B) dialysate concentrations in rats given systemic L-DOPA. Dialysates were collected, at 20 min intervals, in control rats ($n=4$) and in rats ($n=4$) given L-DOPA 25 mg kg^{-1} i.p. twice at 12 h intervals. Baseline L-DOPA and L-DOPA semiquinone concentrations were detected 1 h after last L-DOPA administration. In a third group of four rats given systemic L-DOPA, dialysates were collected for 220 min during continuous intrastriatal infusion of NAC 0.1 mM (horizontal black bar). Values are given as mean± s.e.mean. *$P<0.05$ compared with baseline values: +$P<0.05$ compared with systemic L-DOPA.

220 min) significantly increased L-DOPA dialysate concentrations (up to 5 fold), as compared with baseline values (Figure 3A); in addition, the L-DOPA semiquinone formation was significantly inhibited, as compared with systemic L-DOPA-treated group (Figure 3B).

Effects of intrastriatal L-DOPA infusion on dialysate concentrations of DA, DOPAC + HVA, uric acid and ascorbic acid

Systemic L-DOPA decreased dialysate concentrations of DA, as compared with controls (Table 1). In contrast, continuous intrastriatal L-DOPA infusion (1 μM) significantly increased DA dialysate concentrations (Figure 4A), as compared with baseline values. In addition, L-DOPA infusion slightly though not significantly increased DOPAC + HVA (Figure 4B) and uric acid (Figure 4C). The increase, however, reached statistical significance (ANOVA $P<0.005$) if calculated as percentage of baseline values. Also, ascorbic acid dialysate concentration, which was decreased by systemic L-DOPA (Table 1), slightly increased (Figure 4C) following L-DOPA infusion. The increase reached statistical significance (ANOVA $P<0.005$) if calculated as percentage of baseline values.

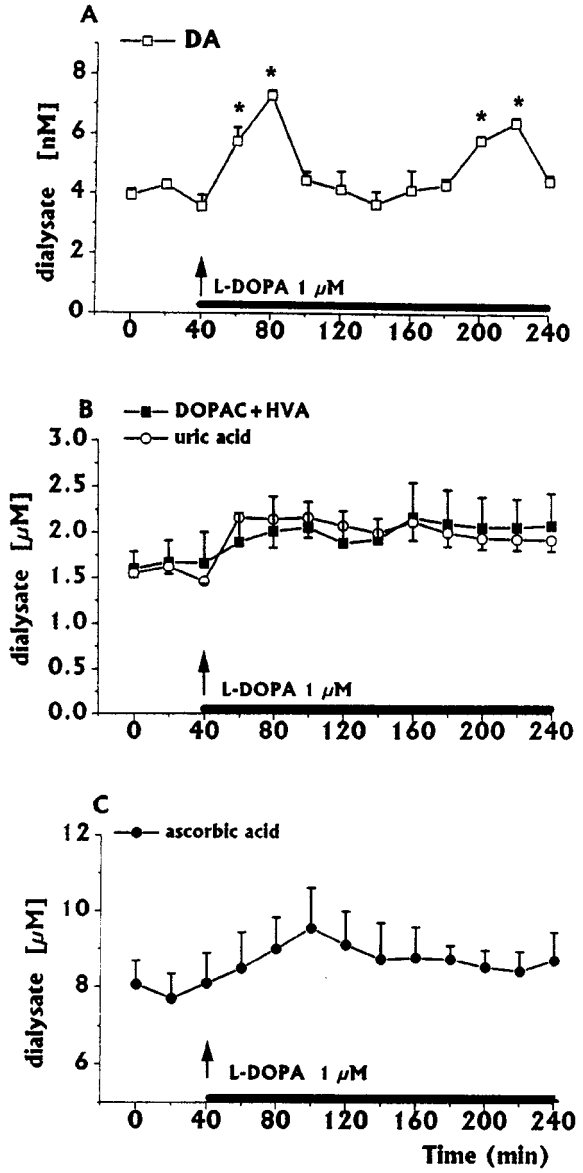

Figure 4 Effect of intrastriatal infusion of L-DOPA on DA (A), DOPAC + HVA and uric acid (B), and ascorbic acid (C) dialysate concentrations. Same rats as in Figure 2. Dialysates were collected, at 20 min intervals, for 200 min during continuous intrastriatal infusion of L-DOPA 1 μM (horizontal black bar). Values are given as mean ± s.e.mean ($n=3$). *$P<0.05$ compared with baseline values.

Effects of intrastriatal NAC infusion on dialysate concentrations of DA, DOPAC + HVA, uric acid and ascorbic acid in L-DOPA-pretreated rats

In rats ($n=4$) given systemic L-DOPA, NAC infusion significantly increased DA dialysate concentrations (Figure 5A), as compared with baseline values. In addition, NAC infusion decreased DOPAC + HVA (Figure 5B) and uric acid dialysate concentrations (Figure 5C), while ascorbic acid concentration was unaffected (Figure 5D).

Effect of intrastriatal $MnCl_2$ infusion on L-DOPA auto-oxidation

The intrastriatal infusion of $MnCl_2$ (30 mM for 15 min) in controls ($n=4$) did not affect L-DOPA levels (Figure 6A); in addition, the L-DOPA semiquinone could not be detected following $MnCl_2$ infusion. In rats given systemic L-DOPA ($n=4$), the intrastriatal infusion of $MnCl_2$ (30 mM for 15 min) significantly decreased L-DOPA concentrations (Figure 6A) and increased dialysate L-DOPA semiquinone (Figure 6B), compared with baseline values. The infusion of NAC (0.1 mM) 20 min before $MnCl_2$ significantly inhibited both manganese-induced decreases in L-DOPA (Figure 6A) and increases in L-DOPA semiquinone (Figure 6B) dialysate concentrations.

When $MnCl_2$ (30 mM for 15 min) was co-infused intrastriatally with L-DOPA (same group of rats shown in Figure 2, see above), dialysate concentrations of L-DOPA semiquinone significantly increased, while those of L-DOPA decreased (Figure 7), as compared with preceding concentrations. Individual dialysate concentrations of L-DOPA were negatively correlated with those of L-DOPA semiquinone (r values range between -0.941 and -0.990, $P<0.0001$, df = 6).

Effects of striatal $MnCl_2$ infusion on dialysate concentrations of DA, DOPAC + HVA, ascorbic acid and uric acid in L-DOPA-pretreated rats

The intrastriatal infusion of $MnCl_2$ (30 mM for 15 min) in untreated rats ($n=4$) induced an early and short-lasting 3 fold increase in DA dialysate concentration, compared with baseline level (Figure 8A), and a late decrease (ANOVA $P<0.005$) in DOPAC + HVA concentrations (Figure 8B). Uric acid concentration increased (ANOVA $P<0.02$) (Figure 8C) while ascorbic acid was unaffected (ANOVA $P>0.7$) (Figure 8D). In rats given systemic L-DOPA ($n=4$), intrastriatal infusion of $MnCl_2$ (30 mM for 15 min) induced an increase in DA dialysate concentration much greater than controls (up to 100 fold baseline level) (Figure 8A). Preliminary experiments showed that manganese releasing effect was only attenuated in Ca^{2+}-free Ringer solution (data not shown).

$MnCl_2$ infusion induced a biphasic change in dialysate DOPAC + HVA concentrations: an initial short-lasting increase followed by a significant and long-lasting decrease, compared with baseline levels (Figure 8B). Uric acid concentrations were further increased (ANOVA $P<0.01$) (Figure 8C), while those of ascorbic acid were unaffected (Figure 8D).

The previous infusion of NAC (0.1 mM) attenuated $MnCl_2$-induced increases in dialysate DA (40 fold baseline level)

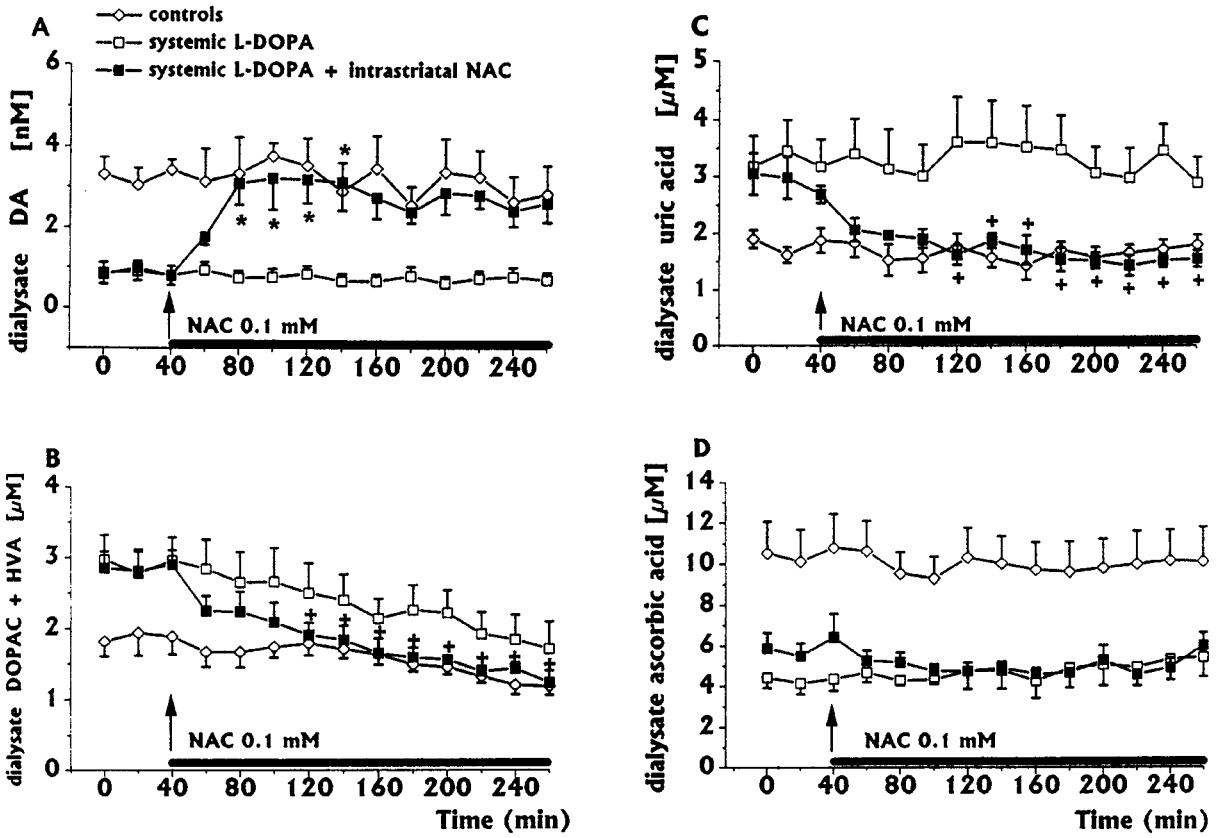

Figure 5 Effect of intrastriatal infusion of NAC on DA (A), DOPAC + HVA (B), uric acid (C) and ascorbic acid (D) dialysate concentrations in rats given systemic L-DOPA. Same groups of rats as in Figure 3. Dialysates were collected, at 20 min intervals, in control rats ($n=4$) and in rats ($n=4$) given L-DOPA 25 mg kg^{-1} i.p. twice at 12 h intervals. Baseline concentrations of neurochemicals were determined 1 h after last L-DOPA administration. In a third group of four rats given systemic L-DOPA, dialysates were collected for 220 min during continuous intrastriatal infusion of NAC 0.1 mM (horizontal black bar). Values are given as mean ± s.e.mean. *$P<0.05$ compared with baseline values: +$P<0.05$ compared with systemic L-DOPA.

(Figure 8A); in addition, NAC infusion antagonized $MnCl_2$-induced changes in DOPAC + HVA (Figure 8B) and increases in uric acid dialysate concentrations (Figure 8C), while ascorbic acid was unaffected (Figure 8D).

Discussion

L-DOPA long-term therapy is useful in relieving Parkinsonian symptoms. L-DOPA, however, being a symptomatic drug, fails to prevent the progressive disabling course of the disease; in addition, associated side-effects increase in frequency and intensity with time (Marsden & Parker, 1977). The results of this study have shown that both systemically and locally administered L-DOPA undergo auto-oxidation to L-DOPA semiquinone in the extracellular space of the rat striatum. In this study, however, we were not able to follow the *in vivo* fate of L-DOPA semiquinone [further oxidation to L-DOPA *o*-quinone, its cyclization and/or conjugation (Shen & Dryhurst, 1998)]. Auto-oxidation of L-DOPA given either systemic or intrastriatal was greatly inhibited by the antioxidant NAC, with a consequent increase in L-DOPA bioavailability. These findings may be of relevance to the long-term L-DOPA therapy of Parkinson's disease. It has been shown that the use of antioxidant tocopherol failed to delay the onset of disability in untreated patients with Parkinson's disease (The Parkinson Study Group, 1993). The effects of co-administering anti-oxidant with L-DOPA in the progression of Parkinson's disease, however, were not investigated in the above trial. On the basis of the present findings, the association of L-DOPA with appropriate antioxidants could be taken in consideration to improve L-DOPA bioavailability, similar to the use of carbidopa or benzserazide co-administrating with L-DOPA. In reality, Spencer *et al.* (1998) have shown that an acceleration of L-DOPA/DA oxidation occurs in Parkinson's disease, probably related to therapy with L-DOPA.

The superoxide radical (O_2^{-}) formation consequent to L-DOPA auto-oxidation (Parson, 1985; Basma *et al.*, 1995) in the extracellular space may account for the decrease in DA concentration induced by systemic L-DOPA, according to the following scheme (Jellinger, 1999): DA + O_2^{-} + $2H^+$ → DA semiquinone + H_2O_2. In fact, the decrease in dialysate DA concentration appears to be the consequence of an increased rate of extracellular DA auto-oxidation rather than a decrease in neuronal content of DA, since the infusion of NAC significantly restored dialysate DA level. Moreover, an increase in formation of 5-S-cysteinyl-DA (which was not detectable by our HPLC-EC method) may be taken into account as an additional mechanism of DA dialysate decrease (Shen & Dryhurst, 1998). In contrast with systemic injection, intrastriatal infusion of L-DOPA induced a significant 2 fold increase in striatal dialysate DA levels. The increase is likely to result from the biotransformation of L-DOPA to DA (Sarre *et al.*, 1998); it is plausible that the newly formed DA is protected from both non-enzymatic oxidation and adducts formation by the efficiency of the extracellular antioxidant system.

ROS formation consequent to L-DOPA auto-oxidation may account also for the decrease in dialysate ascorbic acid

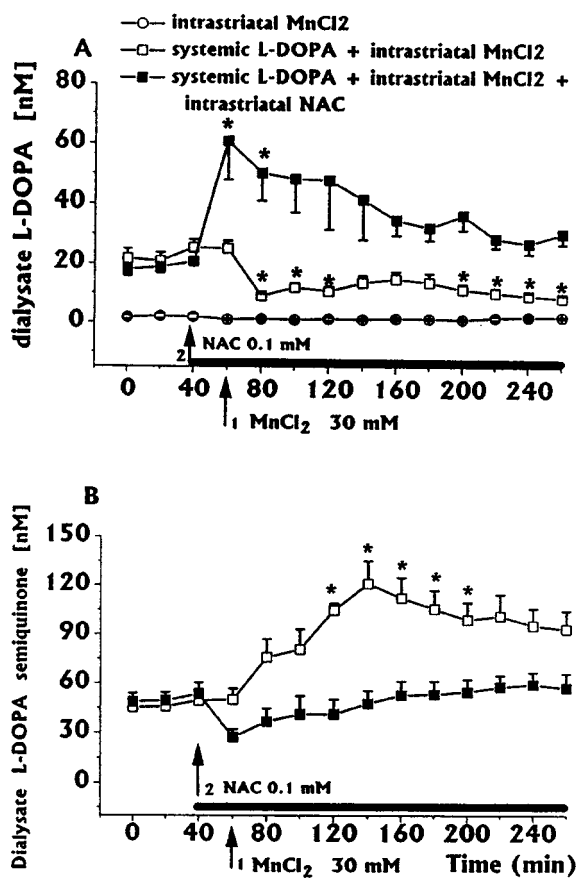

Figure 6 Effect of intrastriatal infusion of $MnCl_2$ on L-DOPA (A) and L-DOPA semiquinone (B) dialysate concentrations in rats given systemic L-DOPA. Dialysates were collected, at 20 min intervals, in control rats ($n=4$) given $MnCl_2$ alone and in rats ($n=4$) pretreated with L-DOPA 25 mg kg^{-1} i.p. twice at 12 h intervals. Baseline L-DOPA and L-DOPA semiquinone concentrations were detected 1 h after last L-DOPA administration. $MnCl_2$ 30 mM was infused for 15 min (arrow 1). In a third group of four rats given systemic L-DOPA, the infusion of $MnCl_2$ (30 mM for 15 min, arrow 1) was done during continuous intrastriatal infusion of NAC 0.1 mM (horizontal black bar, arrow 2). Values are given as mean ± s.e.mean. $^*P<0.05$ compared with baseline values.

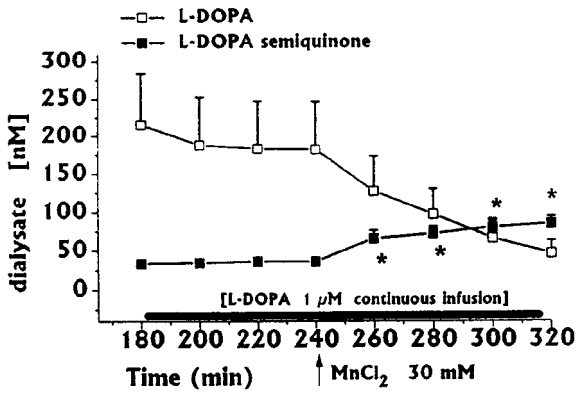

Figure 7 Effect of intrastriatal co-infusion of $MnCl_2$ with L-DOPA on L-DOPA and L-DOPA semiquinone dialysate concentrations (same group of rats as in Figure 2). Dialysates (four samples) were collected, at 20 min interval, after $MnCl_2$ 30 mM infusion (arrow) for 15 min during continuous intrastriatal infusion of L-DOPA 1 μM (horizontal black bar). Values are given as mean ± s.e.mean ($n=3$). $^*P<0.05$ compared with preceding baseline values.

concentration following systemic L-DOPA. Ascorbic acid scavenges ROS generated from catecholamine oxidation (Hastings *et al*., 1996; Lai & Yu, 1997) and inhibits manganese-catalyzed DA auto-oxidation (Florence & Stauber, 1989). Ascorbic acid is not synthesized in the brain. However, it is found in high concentrations throughout the mammalian brain. Ascorbic acid is supplied by active uptake at the choroid plexus site (Spector, 1982), by a carrier-mediated saturable process (Lam & Daniel, 1986) and by simple diffusion at the blood-brain barrier site (Lam & Daniel, 1986). A homeostatic mechanism regulating extracellular brain ascorbic acid has been demonstrated *in vivo* (Miele & Fillenz, 1996). We showed previously (Migheli *et al*., 1999) that ascorbic acid, on equimolar basis, was less active than NAC in protecting PC12 cells from L-DOPA and manganese toxicity. In the present study, baseline striatal dialysate concentrations of ascorbic acid were lowered to 50% of controls following systemic L-DOPA. NAC infusion failed to restore ascorbic acid concentration but restored DA concentration. The decrease in striatal dialysate ascorbic concentration, following systemic L-DOPA, might be a consequence of a widespread ROS formation following L-DOPA auto-oxidation, which might have exceeded capacity of both homeostatic and recycling enzymatic mechanisms. When L-DOPA was infused intrastriatally, ascorbic acid dialysate concentration even increased, probably owing to the efficiency of both homeostatic and recycling enzymatic mechanisms. Studies are in progress to clarify these points.

Changes in DOPAC, HVA and uric acid dialysate levels may result from the diffusion (Basma *et al*., 1995) inside the neuron of superoxide radicals generated from L-DOPA auto-oxidation. These radicals would then promote enzymatic oxidation of both xanthine and DA, according to the following scheme [from Chiueh (1994) and Basma *et al*. (1995), modified]:

$$\text{L-DOPA} + O_2 \rightarrow \text{DSQ} + O_2\rightarrow + \text{DA(MAO)} \rightarrow \text{DOPAC} + \text{ROS}$$
$$\text{L-DOPA} + O_2 \rightarrow \text{DSQ} + O_2\rightarrow + \text{xanthine(XO)} \rightarrow \text{uric acid} + \text{ROS}$$

where $DSQ^{\cdot}$ is the semiquinone free radical of L-DOPA. The inhibition of L-DOPA auto-oxidation by NAC infusion resulted in a decrease of enzymatic oxidation of both xanthine and DA, with a consequent decrease in dialysate concentrations of the oxidation products, respectively uric acid and DOPAC.

The role of uric acid deserves some attention. Uric acid is an active component of the neuronal antioxidant pool (Jellinger, 1999). It is capable of inhibiting free-radical initiated lipid peroxidation and DNA damage (Cohen *et al*., 1984); in addition, it forms strong complexes with iron, particularly Fe^{3+}. According to Sevanian *et al*. (1991), one of uric acid scavenging activity is to maintain ascorbic acid in its reduced form in biological fluids. We have shown that inhibition *in vivo* of uric acid production by means of allopurinol greatly decreases extracellular ascorbic acid concentration in the rat striatum (Enrico *et al*., 1997). Church & Ward (1994) found significantly lower levels of uric acid in the *substantia nigra* of Parkinsonian patients, while ascorbic acid levels were in the range of control values; moreover, they found that DA auto-oxidation rate was enhanced in caudate and *substantia nigra* homogenates of Parkinsonian patients, compared to controls; the addition of the uric acid or uricase to the brain homogenates slowed and, respectively, increased DA auto-oxidation rate. These data outline the role of uric acid as ROS scavenger in protecting both DA and AA from oxidation.

Manganese intrastriatal infusion in L-DOPA pre-treated rats decreased dialysate DOPA concentrations and greatly increased those of L-DOPA semiquinone. The increase in L-DOPA auto-oxidation may account for the manganese

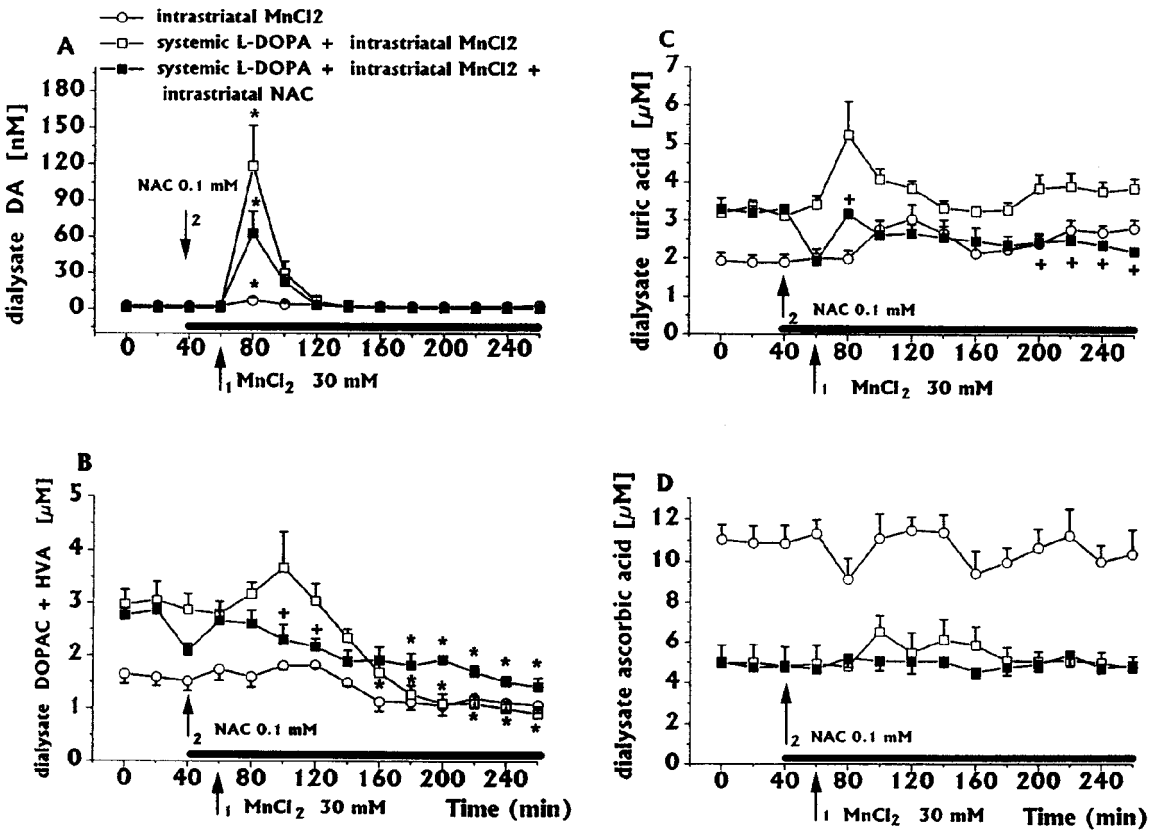

Figure 8 Effect of intrastriatal infusion of $MnCl_2$ on DA (A), DOPAC + HVA (B), uric acid (C) and AA (D) dialysate concentrations in rats given systemic L-DOPA. Same groups of rats in Figure 6. Dialysates were collected, at 20 min intervals, in control rats ($n=4$) given $MnCl_2$ alone and in rats ($n=4$) pretreated with L-DOPA 25 mg kg^{-1} i.p. twice at 12 h interval. Baseline neurochemical concentrations were detected 1 h after last L-DOPA administration. $MnCl_2$ 30 mM was infused for 15 min (arrow 1). In a third group of four rats given systemic L-DOPA, the infusion of $MnCl_2$ was done during continuous intrastriatal infusion of NAC 0.1 mM (horizontal black bar, arrow 2). Values are given as mean ± s.e.mean. $^*P<0.05$ compared with baseline values: $+P<0.05$ compared with systemic L-DOPA + intrastriatal $MnCl_2$.

enhancing effect *in vitro* on L-DOPA toxicity to PC12 cells (Migheli *et al*., 1999); moreover, the NAC inhibition of manganese-induced increase in L-DOPA auto-oxidation may account for NAC protective effect *in vitro*. However, the manganese-induced changes in DA release and metabolism underlie additional mechanisms. It has been shown that manganese, when injected in the rat striatum, is subjected to widespread axonal transport, reaching in a few hours the ipsilateral *substantia nigra*, thalamus and hypothalamus (Takeda *et al*., 1998). Sloot *et al*. (1996) found that manganese, when given intrastriatally to rats, induced an early depletion of DA (within 2 h) and a relatively late formation of hydroxyl radicals. In the present study, intrastriatal infusion of manganese in untreated rats induced an immediate and short-lasting increase in DA release (3 fold baseline level) and a late decrease in DOPAC + HVA concentrations. In rats pretreated with systemic L-DOPA, intrastriatal manganese infusion elicited a greater increase in DA release (100 fold baseline level). In addition, manganese decreased dialysate DOPAC + HVA concentration following a short-lasting early increase. In the previous study *in vitro* (Migheli *et al*., 1999), we have shown that both DA formation and metabolism were impaired in PC12 cells exposed to L-DOPA associated with manganese. In the present study, there is no direct evidence of a manganese induced-depletion of striatal DA; however, since newly synthesized DA is the main substrate for MAO (Zetterström *et al*., 1986), the last decrease in DOPAC + HVA dialysate concentrations is likely to be a consequence of manganese-induced depletion of newly synthesized DA.

Exposure to manganese in drinking water is not a risk factor for idiophatic Parkinson's disease (Vieregge *et al*., 1995) and serum and urinary excretion of manganese are apparently unrelated to the risk of developing Parkinson's disease (Jimenez-Jimenez *et al*., 1995). However, the fact that manganese as a transition metal greatly increased L-DOPA auto-oxidation *in vivo*, may be of relevance to the potential toxicity of L-DOPA long-term therapy to the nigro-striatal system. Misregulation of iron metabolism and iron-induced oxidative stress are widely believed to be important pathogenetic mechanism of neuronal death in Parkinson's disease (Jellinger, 1999). Superoxide radical releases iron from storage proteins and enzyme [4Fe-4S] clusters (Keyer & Imlay, 1996). Iron, a transition metal, is known to promote L-DOPA and DA auto-oxidation *in vitro* (Spencer *et al*., 1998). It is likely that this effect might also occur *in vivo*. In a study in progress on iron/L-DOPA interaction in the striatum of the freely moving rat, preliminary results showed that intrastriatal co-infusion of L-DOPA and iron (II) lowers both L-DOPA and DA dialysate concentrations, as a result of an increased auto-oxidation of both catechols (unpublished observations).

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