Research report

Time-course and dose–response study on the effects of chronic L-DOPA administration on striatal dopamine levels and dopamine transporter following MPTP toxicity

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Abstract

Despite a long-lasting therapeutic use of L-DOPA in Parkinson’s disease, doubts still remain concerning the possibility that chronic L-DOPA might accelerate the progression of this movement disorder. To address this point, in the present study we examined the effects of chronic L-DOPA administration either in intact or MPTP-treated parkinsonian mice. We produced an intermediate striatal dopamine loss by administering a low dose of MPTP (30 mg/kg); then, we treated mice chronically, for different time intervals, with a daily dose of L-DOPA (50 mg/kg). In particular, to study the time-course of the effects of L-DOPA on the recovery of nigrostriatal dopamine axons, mice were sacrificed at 5, 30, 60, and 90 days after a daily L-DOPA administration. To evaluate presynaptic integrity of the nigrostriatal pathway we measured dopamine, metabolite levels, and dopamine uptake sites. In the same animals, we measured striatal serotonin levels and we analysed monoamine content in the olfactory bulb. Administration of MPTP produced a neurotoxic effect, which fully recovered in 2–3 months. Daily L-DOPA administration did not modify this recovery process. Additionally, there was no significant effect of L-DOPA in intact mice, despite a slight decrease in striatal dopamine levels at 5 and 30 days. However, this effect was neither worsened nor reproduced by administering higher doses of L-DOPA (up to 400 mg/kg) for the same amount of time. These data rule out neurotoxic effects induced by prolonged L-DOPA administration, both in intact and MPTP-treated mice. Moreover, administration of L-DOPA does not change the recovery process which takes place after a nigrostriatal lesion. © 2000 Elsevier Science B.V. All rights reserved.

Theme: Disorders of the nervous system

Topic: Degenerative disease: Parkinson’s

Keywords: L-DOPA; MPTP; Recovery; Experimental parkinsonism; DAT

1. Introduction

Since its introduction in the 1960s (see Ref. [18]), the neurotransmitter precursor L-DOPA, via its decarboxylation to dopamine (DA), represents the most effective therapy in Parkinson’s disease (PD) [19]. There is concern, however, that the symptomatic relief induced by L-DOPA conveys a toxic effect by accelerating the neuronal loss which normally takes place during the disease process [19]. Indeed, there is no doubt that, after prolonged L-DOPA administration, a number of neurological complications (i.e., dyskinesia), tightly related to the ongoing therapy occur [40,4]. It has been postulated that even the natural course of the disease might be accelerated by chronic L-DOPA administration [15]. Thus, an open question still remains concerning L-DOPA-related symptoms: do they represent a functional and potentially reversible behavioral disorder, or do they reflect an actual progression of the disease, substantiated by accelerated neuron and/or axon terminal loss promoted by L-DOPA?

This latter hypothesis was suggested since the late 1970s [15], when it was demonstrated that L-DOPA produces oxidative compounds like semiquinone and O-quinone.
derivatives which might be toxic for DA neurons. In line with this, a number of in vitro and microinfusion studies carried out during the last decade, provided evidence for l-DOPA- and/or DA-induced neurotoxicity in non-catecholamine [36,7,27] and catecholamine cell lines [22,29,39], in primary mesencephalic cell cultures [23,24,30], and via direct microinfusion of DA within the striatum [10].

Despite these results, obtained with in vitro (and microinfusion) studies, in vivo experiments consistently failed to demonstrate neurotoxic effects induced by l-DOPA on intact nigrostriatal DA system [17,31].

These results are in line with neuropathological studies carried out in humans with a misdiagnosis of PD who, despite receiving a high daily dose of l-DOPA for several years, showed a normal mesencephalic histopathology [32,33].

However, it should be considered that during PD, exogenous l-DOPA is handled differently by the few surviving DA neurons. For instance, during the progression of the disease only scant striatal DA axons are taking up the same amount of l-DOPA, which therefore, reaches a higher concentration in each nigrostriatal terminal. Moreover, in PD surviving DA axons undergo an increased neurotransmitter metabolism [1,21] which leads to augmented rate of l-DOPA utilisation and, thereby, to heightened DA oxidation. These effects might converge in increasing oxidative stress within surviving DA terminals accelerating the progression of PD [38].

Indeed, a few in vivo experiments carried out in parkinsonian animals provided evidence for a neurotoxic effect of l-DOPA [5,28]; however, in other studies this toxic effect was not observed [9,25]. In particular, in a recent elegant report, Murer et al. [25] demonstrated the absence of l-DOPA toxicity, suggesting a trophic role of l-DOPA administered for 6 months to rats carrying a partial striatal DA loss induced by 6-hydroxydopamine (6-OHDA).

In the present study, we examined the effects of chronic l-DOPA administration in parkinsonian mice by using a different experimental approach. We administered l-DOPA chronically to mice carrying a partial lesion of the nigrostriatal DA pathway induced by the neurotoxin MPTP. The experiment was performed in mice since, among rodents, this animal species is more sensitive to experimental Parkinsonism induced by DA neurotoxins [14]. In order to evaluate either potential deleterious, or protective effects induced by chronic l-DOPA, we produced an intermediate dose of MPTP and then received a daily chronic administration of l-DOPA methyl ester (6-OHDA).

In the present study, we examined the effects of chronic l-DOPA administration in parkinsonian mice by using a different experimental approach. We administered l-DOPA chronically to mice carrying a partial lesion of the nigrostriatal DA pathway induced by the neurotoxin MPTP. The experiment was performed in mice since, among rodents, this animal species is more sensitive to experimental Parkinsonism induced by DA neurotoxins [14]. In order to evaluate either potential deleterious, or protective effects induced by chronic l-DOPA, we produced an intermediate striatal DA loss by administering a low dose (30 mg/kg) of MPTP. Then, we challenged these animals with a daily dose of l-DOPA (50 mg/kg). Mice were sacrificed at four different time intervals (5, 30, 60, and 90 days) after MPTP administration. Apart from disclosing a potential effect depending on the time of exposure to l-DOPA, this experimental protocol was designed to evaluate the time-course of potential effects of l-DOPA on the recovery of nigrostriatal DA terminals. In mice, in 2–3 months after the onset of a moderate striatal DA loss, a spontaneous recovery of nigrostriatal DA axons takes place [12]. To document the presynaptic integrity of the DA nigrostriatal pathway we measured both DA, metabolite levels, and DA uptake sites both in intact and MPTP-lesioned striatum, injected chronically either with saline or l-DOPA. To evaluate the site-specificity of the effects induced by MPTP and/or l-DOPA we also analysed monoamine levels in the olfactory bulb.

2. Materials and methods

2.1. Animals

Two hundred and sixty C57 Black male mice (Charles River Calco, CO, Italy), 9 weeks old, were used for the study. Since previous experiments demonstrated that MPTP toxicity critically depends on age [3,8], all mice used in the present work were 9 weeks old at the time of MPTP administration. Mice were housed with free access to food and water and kept under environmentally controlled conditions (12-h light/dark cycle with light on between 07:00 and 19:00). In the present study, we evaluated the potential toxicity of l-DOPA: it is established that DA neurotoxins (i.e., amphetamines) vary their efficacy depending on housing conditions (for a review, see Ref. [35]); therefore, we did not vary the number of animals per cage (n=10) and the size of the cages (38×22 cm wide and 15 cm high).

Experiments were approved by Local Ethical Committee and animals were treated in accordance with the Guidelines for Animal Care and Use of the National Institutes of Health.

2.2. Experimental design

2.2.1. Time-course study

In the time-course study, 160 C57 Black mice were divided into four groups each composed of 40 animals receiving various treatments: Group A received an injection of saline and then again, it was administered daily with saline. Group B was administered saline once and then, chronically a daily dose of l-DOPA methyl ester hydrochloride (Research Biochemicals, RBI, Natick, MA; 50 mg/kg, free base). Group C was treated with a single dose of MPTP hydrochloride (RBI; 36 mg/kg, corresponding to 30 mg/kg of MPTP), then it was administered daily either with saline or l-DOPA. To evaluate the site-specificity of the effects induced by MPTP and/or l-DOPA we also analysed monoamine levels in the olfactory bulb.
cleared from the striatum [11]. The time-course of the recovery process of striatal DA innervation following an intermediate dose of MPTP was evaluated sacrificing mice from each group at four distinct time intervals. In particular, the original four groups of mice were further divided into four subgroups each composed of 10 animals which were sacrificed at 5 days, and 1, 2, and 3 months, respectively, after MPTP administration.

During chronic l-DOPA administration mice were weighted once a week in order to maintain a constant amount of l-DOPA/body weight. Injections were carried out intraperitoneally (i.p.), using a constant volume (10 ml/kg). Mice were sacrificed at 40 h after the last l-DOPA/saline injection and their brains were immediately removed.

The left striatum and the olfactory bulb were dissected and processed for measuring monoamine levels, whereas the right striatum from the same animals was dissected and processed for measuring presynaptic striatal DA uptake sites.

2.2.2. Dose–response study
Since in the time-course study (see Section 3) we observed a slight decrease of striatal DA levels at early time intervals (5 days and 1 month) after l-DOPA (50 mg/kg) administration to intact mice, we explored this effect in detail by increasing the dosage of l-DOPA. In this additional experimental step, 100 C57 Black mice were divided into five groups each composed of 20 mice. Group A received saline. Group B was administered with l-DOPA at 50 mg/kg. Group C received l-DOPA at 100 mg/kg. Group D was administered with l-DOPA at 200 mg/kg. Group E was injected with l-DOPA at 400 mg/kg. From each group, 10 animals were sacrificed at 5 days after a daily saline/l-DOPA administration, whereas 10 mice were sacrificed after 1 month of daily treatment. In these mice we measured striatal monoamine levels.

2.3. Assay of monoamines
The striatum and the olfactory bulb were sonicated in 0.6 ml of ice-cold 0.1 M perchloric acid containing 10 ng/ml of 3,4-dihydroxybenzylamine (DBA, Sigma, San Louis, MO) as the internal standard. An aliquot of the homogenate (50 µl) was assayed for proteins [20]. After centrifugation at 8000×g for 10 min, 20 µl of the clear supernatant were injected into an HPLC system where DA, norepinephrine (NE), serotonin (5-HT) and metabolites were analysed as previously described [12].

2.4. Measurement of striatal DA uptake sites
For radioligand binding measurement of the plasma membrane DA transporter (DAT) the striatum was quickly removed, frozen in liquid nitrogen and kept at −80°C until assayed. For each assay one striatum was homogenised in 1.5 ml of ice-cold buffer (50 mM Tris, 5 mM EDTA, 320 mM sucrose; pH 7.5) and centrifuged at 1000×g for 10 min at 4°C. The pellet was discarded and the supernatant centrifuged at maximum speed in a microfuge for 30 min at 4°C. The pellet was resuspended in 1.2 ml of binding buffer (50 mM Tris, 300 mM NaCl, 5 mM, 0.1% ascorbic acid; pH 7.9) by sonication. After addition of [3H]GBR 12935 (DuPont NEN; final concentration 3 nM, 40–60 Ci/mmole), membrane preparations were incubated for 45 min at 25°C. Non-specific binding was determined in the presence of DA 200 mM. Incubation was stopped by centrifugation for 30 min. The pellet was washed twice with 1 ml of binding buffer and radioactivity was measured by scintillation counting.

2.5. Data analysis
For monoamine assay, a standard curve was prepared using known amounts of DA, NE, 5-HT and metabolites (Sigma), dissolved in 0.1 M perchloric acid containing a constant amount (10 ng/ml) of the internal standard (DBA), as used for tissue samples. The standard curve for each compound (DA, NE, 5-HT or metabolites) was calculated using regression analysis of the peak areas for known concentrations of each compound. For binding studies data are expressed as percentage of specific binding; the latter was calculated from the ratio between the cpm/mg protein of specific binding, and the cpm/mg protein of total binding. For NE, DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-HT and 5-hydroxy-indoleacetic acid (5-HIAA) levels, results are expressed as the mean±S.E.M. of eight to 10 animals per group. For binding studies, results are expressed as the mean±S.E.M. of eight to 10 animals per group. Effects of MPTP and/or l-DOPA on monoamine levels in the striatum and olfactory bulb as well as on striatal GBR 12935 binding sites, were evaluated using analysis of variance with Sheffe’s post-hoc analysis.

3. Results
3.1. Effects of MPTP administration on the time-course of striatal DA and the DA transporter
As shown in Fig. 1A, 5 days after administration of the neurotoxin MPTP (30 mg/kg) we measured an intermediate degree of striatal DA loss at 5 days compared with control values. This effect became less evident at prolonged time intervals. In particular, at 1 month there was an increase in DA levels in MPTP-treated mice, which became closer to control values. At 3 months, there were no longer significant differences between MPTP-treated mice and controls. A similar trend was observed for the DA metabolites DOPAC (Fig. 1B) and HVA (Fig. 1C),
although the depleting effect of MPTP for DA metabolites was less pronounced. Levels of 5-HT and its metabolite 5-HIAA within the striatum were not affected by MPTP administration at any time interval (data not shown). No significant difference was measured in saline-injected controls during the 3 months observation time (Fig. 1). As shown in Table 1, no significant effect was produced in the olfactory bulb by MPTP administration at any time interval compared with controls. Similarly, in the olfactory bulb MPTP administration did not modify NE levels (Table 1).

As shown in Fig. 2, measurement of striatal plasma membrane DAT matched the data obtained by assaying DA levels (Fig. 1A). Five days after MPTP administration there was an intermediate decrease in DA binding sites compared with controls (saline, 18.39±2.59%; MPTP, 8.25±2.46%), whereas at 2 and 3 months striatal DAT levels in MPTP-treated mice were close to controls.

### 3.2. Effects of L-DOPA per se

As shown in Fig. 1A, chronic administration of L-DOPA did not modify striatal DA levels. At 5 days and 1 month after daily L-DOPA administration there was a slight decrease in striatal DA levels; however, in the following time intervals, this effect was no longer present (Fig. 1A). Similarly, striatal DA uptake sites were not affected by chronic L-DOPA administration (Fig. 2). Neither striatal 5-HT levels (data not shown) nor levels of monoamines in the olfactory bulb (Table 1) were modified during the time-course of L-DOPA administration compared with controls.

### Table 1

Monoamine and metabolite levels (values are given in ng/mg protein) in the olfactory bulb at different time intervals.

<table>
<thead>
<tr>
<th></th>
<th>5 days</th>
<th>30 days</th>
<th>60 days</th>
<th>90 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA</td>
<td>4.23±0.69</td>
<td>3.44±0.49</td>
<td>4.20±0.45</td>
<td>4.01±0.28</td>
</tr>
<tr>
<td>MPTP</td>
<td>3.92±0.50</td>
<td>4.04±0.37</td>
<td>4.74±0.71</td>
<td>3.90±0.57</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>4.36±0.64</td>
<td>3.80±0.39</td>
<td>4.40±0.37</td>
<td>4.02±0.29</td>
</tr>
<tr>
<td>L-DOPA+MPTP</td>
<td>3.85±0.70</td>
<td>4.23±0.48</td>
<td>3.94±0.57</td>
<td>4.39±0.62</td>
</tr>
</tbody>
</table>

Male C57 Black mice have been sacrificed at different time intervals after a single MPTP (30 mg/kg) administration following a chronic daily dosage of either L-DOPA (50 mg/kg i.p.) or an equivalent volume of saline. Male C57 Black mice were sacrificed at different time intervals (5, 30, 60, 90 days) after an i.p. injection of MPTP (30 mg/kg) and a daily administration of L-DOPA (50 mg/kg i.p.). Mice were sacrificed, the left striata were dissected and processed for HPLC analysis. The levels of dopamine (DA) (A), dihydroxyphenylacetic acid (DOPAC) (B), and homovanillic acid (HVA) (C) were evaluated. For each time interval results were obtained from eight to 10 animals per group and are given as the mean±S.E.M. At each time interval differences between groups were evaluated using ANOVA with Sheffe’s post-hoc analysis. *P<0.05 compared with controls.
3.3. Effects of higher doses of L-DOPA

Since at 5 days and 1 month there was a slight decrease in DA levels of L-DOPA-treated mice, we increased the daily dose of L-DOPA up to 8-fold. As shown in Fig. 3, no effects were observed either at 5 days (Fig. 3A) and 1 month (Fig. 3B) after a daily administration of L-DOPA up to 400 mg/kg.

3.4. Effects of a daily administration of L-DOPA on the recovery following MPTP-induced neurotoxicity

Animals injected with MPTP and then receiving a daily L-DOPA administration had the same striatal DA levels as MPTP-treated animals receiving a chronic administration of saline (Fig. 1). This was confirmed by assaying DAT binding sites (Fig. 2). Therefore, L-DOPA administration did not modify the spontaneous recovery process occurring in partially lesioned nigrostriatal DA terminals (Figs. 1 and 2). In this case, we did not observe the slight decrease in DA levels which was detected at early time intervals in intact mice injected with L-DOPA. Striatal 5-HT and 5-HIAA levels were not affected at any time interval. Similarly, no effect was detected on monoamine levels in the olfactory bulb (Table 1).

4. Discussion

In the present study we confirmed previous data [11] showing that i.p. administration of the DA neurotoxin MPTP at 30 mg/kg to C57 Black mice produces an intermediate degree of toxicity to the nigrostriatal DA pathway (measured by the amount of striatal DA loss and by the decrease of striatal DA uptake sites). Following this kind of lesion, a full recovery process takes place in 2–3 months after MPTP administration.

Results of this study show that a single i.p. daily administration of L-DOPA (50 mg/kg), does not affect striatal DA levels when administered to intact mice for different time intervals (5, 30, 60, or 90 days). We observed only a slight decrease (the effect was significant at the ANOVA using the Fisher test but not using Sheffe’s post-hoc analysis) in DA levels after 5 days and 1 month after a daily injection of L-DOPA at the dose of 50 mg/kg. However, this latter effect does not appear to be relevant since: (1) it was not observed when measuring DA uptake sites; (2) it was neither increased nor even reproduced by the administration of higher doses of L-DOPA (up to 400 mg/kg) for the same amount of time.

In the present study we decided to administer L-DOPA as a single, daily i. p. injection for several reasons: (1) to avoid physiological variations in daily L-DOPA intake due to oscillations in the amounts of food/water intake by the animals; (2) to produce a higher concentration of L-DOPA available at the same time in the dorsal striatum; (3) to
evaluate a potential neurotoxic effect induced by a bolus of \textit{l}-DOPA compared with oral administration evaluated in previous studies.

The dose of \textit{l}-DOPA (50 mg/kg) corresponds to a daily administration of 4 g in an adult weighing 80 kg which, given the partial (10\%) penetration of \textit{l}-DOPA within the central nervous system in the absence of a peripheral decarboxylase inhibitor, corresponds to a low therapeutic dosage of the DA precursor (0.4 g/die). However, we did not obtain any effect even increasing the dose of \textit{l}-DOPA up to 400 mg/kg. Nonetheless, the efficacy of 50 mg/kg of \textit{l}-DOPA in modulating striatal DA activity is shown by previous studies in which \textit{l}-DOPA (50 mg/kg) doubled striatal DA release in mice [13].

These results obtained in mice, confirm what previously reported in intact rats [17], where no changes were observed in the nigrostriatal DA system after chronic \textit{l}-DOPA administration. These data are in line with neuropathological studies carried out in patients with a wrong diagnosis of PD who received for several years a high daily dosage of \textit{l}-DOPA. In these patients there was a normal mesencephalic histopathology [32,33].

Therefore, in the present study we did not find any effect after administration of different doses of \textit{l}-DOPA to otherwise intact animals. Furthermore, we found that MPTP-treated mice chronically injected \textit{l}-DOPA had similar striatal DA levels as MPTP-treated mice injected daily with saline. Similarly, measurement of presynaptic DA uptake sites after MPTP administration did not show significant differences between \textit{l}-DOPA- and saline-injected mice. The lack of any deleterious effect of \textit{l}-DOPA was documented both immediately after the production of nigrostriatal DA toxicity by MPTP (animals killed at 5 days after MPTP administration) and during the recovery process measured at 1, 2 and 3 months after the onset of experimental Parkinsonism. The analysis of four different time intervals after the lesion allowed us to detect potential transient effects induced by \textit{l}-DOPA on the recovery process. These data show that even when the nigrostriatal DA pathway is partially damaged, chronic \textit{l}-DOPA supplementation does not produce any deleterious effect on surviving striatal DA terminals. Therefore chronic \textit{l}-DOPA administration did not worsen experimental Parkinsonism either directly, by enhancing the neurotoxic effects of MPTP, or indirectly, by interfering with the spontaneous recovery process.

It has been hypothesised that increased metabolic rate of surviving DA axons [1] would result in marked elevation of rate of intracellular DA oxidation, thereby leading to increased oxidative stress, which in turn, accelerate the progression of PD [38]. Within this context, it has been suggested that chronic treatment with \textit{l}-DOPA, by increasing the substrate for the DA oxidative pathway, might exacerbate oxidative stress. This hypothesis has been strengthened by the finding that exogenous administration of \textit{l}-DOPA results in the formation of hydroxyl radicals in vivo [37]. However, in the present study, this latter effect does not seem to play any role on the survival of DA axons. This is consistent with a recent finding showing that \textit{l}-DOPA administration to 6-OHDA injected rats does not increase oxidative stress [6].

The present data are in line with a previous study in which \textit{l}-DOPA has been administered to MPTP-treated monkeys [34] for up to 11 weeks without modifying striatal DA levels and striatal DA uptake sites. However, in this latter experiment MPTP produced by itself a massive loss of the nigrostriatal DA system (more than 90\%) making it difficult to evaluate a worsening of the DA lesion by \textit{l}-DOPA administration. In the present study, MPTP has been administered at a dose producing an intermediate degree of nigrostriatal DA damage; this was aimed at evaluating either potential deleterious effects, no influence, or, on the other hand, a protective phenomenon. In these experimental conditions we confirmed what recently published by Murer et al. [25] who administered chronically \textit{l}-DOPA to rats underwent an intermediate lesion using the neurotoxin 6-OHDA. In this study the authors did not find any exacerbation of 6-OHDA-induced nigrostriatal damage at 6 months after inducing the lesion. On the other hand, based on measurement of tyrosine hydroxylase (TH) Murer et al. [25] found a beneficial effect following \textit{l}-DOPA administration. In the present experiment we did not find these beneficial effects. This might be due to several reasons: (1) the parkinsonian model we used here (MPTP compared with 6-OHDA); (2) the animals species (mice compared with rats); (3) the maximum amount of time available for the effect to occur (3 months compared with 6 months); (4) the markers used in this study to evaluate the integrity of the nigrostriatal DA pathway: striatal levels of DA and metabolites combined with the measurement of DA transporter (in the present study), compared with striatal TH, vesicular monoamine transporter and DA transporter measured by Murer et al. [25]; (5) the degree of nigrostriatal lesion, which seems to be more marked in the case of the work performed by Murer et al. [25]; (6) the route of administration of \textit{l}-DOPA (i.p. in the present study compared with oral in the case of Murer et al. [25]).

Nonetheless, it should be emphasised that, despite all the above-mentioned experimental differences, our data converge with those of Murer et al. [25] in showing the lack of any deleterious effect following chronic \textit{l}-DOPA both to intact and to parkinsonian animals, even when the drug is administered as a constant i.p. dose.

In conclusion, a chronic daily administration of \textit{l}-DOPA does not worsen nor the integrity neither the recovery of a partially lesioned nigrostriatal pathway. These data, joined with previous studies, confirm what has been already inferred in parkinsonian patients more than a decade ago by Blin et al. [4] and recently commented on by Agid [2]: \textit{l}-DOPA-induced abnormal involuntary movements are only elicited by \textit{l}-DOPA and they are indeed due to an
independent, ongoing disease progression. On the other hand, L-DOPA administration does not seem at all to contribute to the progression of PD. The vast literature showing the in vitro neurotoxic effects of L-DOPA should probably be re-considered carefully, monitoring the dose, the time of exposure and the kind of cell culture. For instance, Mytilineou et al. [26] pointed out that varying in vitro conditions L-DOPA might exert either toxic or protective effects on primary mesencephalic cells. This could explain contradictory findings showing either toxic [23,24] or protective effects [16] of L-DOPA administration to primary mesencephalic neurones.

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