

Research article

L-DOPA treatment in MPTP-mouse model of Parkinson's disease potentiates homocysteine accumulation in substantia nigra



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HIGHLIGHTS

- L-DOPA therapy in Parkinson's disease causes HHcy.
- Hcy is elevated in nigra of L-DOPA treated parkinsonian mouse.
- HHcy in nigra is a potential threat towards vulnerability of dopamine-rich neurons.

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ABSTRACT

One of the intermediates of methionine cycle, the homocysteine (Hcy), elevates in plasma of Parkinson's disease (PD) patients undergoing L-DOPA (3,4-dihydroxyphenylalanine) therapy and has been regarded as a risk factor of the disease. Several evidences pointed out that Hcy causes degeneration of dopaminergic neurons. In rodent, elevated level of Hcy in brain or infusion of the same directly into the substantia nigra (SN) potentiates dopaminergic neurodegeneration. However, the influence of L-DOPA therapy on the levels of Hcy in dopamine-rich regions of the brain (striatum and SN) of experimental models of PD is not known. The present study, for the first time, tested the hypothesis that L-DOPA treatment in experimental mouse model of PD potentiates Hcy accumulation in the dopamine-rich regions of the brain. We found a significant elevation of Hcy level in nigrostriatum in naïve as well as parkinsonian mice as a result of chronic L-DOPA treatment. Interestingly, L-DOPA treatment significantly elevates Hcy level in nigra but not in striatum of parkinsonian mice, when compared with L-DOPA naïve group. However, there is no significant decrease in the number of dopaminergic neurons in SN region in the parkinsonian mice given L-DOPA treatment. Thus, the present study demonstrates that L-DOPA treatment potentiates the level of Hcy in the SN without causing aggravated neurodegeneration in parkinsonian mice model.

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1. Introduction

Parkinson's disease (PD) occurs due to loss of dopamine containing neurons in the substantia nigra (SN) pars compacta region of midbrain [1]. Dopamine replacement therapy with L-3,4-dihydroxyphenylalanine (L-DOPA) has been the gold standard drug for the symptomatic treatment of PD [2]. However, prolonged use of the drug causes adverse side-effects [3,4]. Hyperhomocysteine-

mia (HHcy) is a condition where the concentration of homocysteine (Hcy), an intermediate amino acid of the methionine cycle, is elevated by several folds in plasma [5,6]. Long term L-DOPA therapy in PD patients has been reported to elevate the Hcy level in plasma as well as in cerebrospinal fluid [7–11].

Several studies of the past decade have suggested HHcy as a potential risk factor for PD, owing to its nigral dopaminergic neuron specific toxicity [9,12–15]. In the plasma of naïve rat, L-DOPA-induced HHcy has been reported earlier [16]. In MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) mouse model of PD given L-DOPA treatment, Hcy level in the whole-brain lysate was reported to be elevated [17]. However, the effect of L-DOPA on the levels of Hcy in the dopamine-rich regions of brain in experimental model of PD is not reported yet. Thus, the present study has

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been undertaken to investigate the level of Hcy in nigrostriatum of parkinsonian mice under chronic L-DOPA treatment.

2. Material and methods

2.1. Experimental design

Male Swiss albino mice (23–25 g), used in the present study, were maintained under standard conditions (12 h light/dark cycles, $24 \pm 2^\circ\text{C}$ temperature and $60 \pm 5\%$ humidity) and the experimental protocols were as per National and Institutional guidelines. Mice were treated with MPTP daily for 5 consecutive days, at the dose of 30 mg/kg (i.p.) [18]. Mice were orally administered L-DOPA (250 mg/kg), containing carbidopa (25 mg/kg) as per Borah and Mohanakumar [19] or vehicle (0.9% Sodium Chloride) daily for 28 days from the 5th day post-MPTP injection. Another set of mice were administered with L-DOPA containing carbidopa (250 mg/kg L-DOPA and 25 mg/kg carbidopa; daily) for 28 days from the 5th day post saline treatment. PD model was validated using motor behavioural tests and analysis of DA level in the striatum. Plasma Hcy was estimated at 2 and 24 h after the 28th dose of L-DOPA treatment. For brain Hcy estimation, the animals were sacrificed at 2 h and 24 h after 28th dose of L-DOPA. Striatal dopamine level was analyzed only in MPTP treated animals on the 28th day.

2.2. Motor behavioural tests

Motor behavioural tests, viz., Akinesia, Catalepsy and Swim test, were performed, following Bhattacharjee et al. [20], at the end of MPTP treatment. Briefly, for Akinesia test, the animals were placed over a flat wooden surface and the latency in moving all the four limbs was recorded, after an acclimatization period of 5 min. For Catalepsy test, the hind limbs of animals were placed on a 3 cm high wooden block, and the latency of the animals in moving to the flat surface was recorded. For Swim test, the animals were placed in a tub containing 12 cm high water, maintained at $27 \pm 2^\circ\text{C}$, and their swimming ability was scored for each minute for a total period of 10 min as follows: '3' – for continuous swimming, '2' continuous swimming with occasional floating, '1' for floating with occasional swimming, and '0' for continuous floating.

2.3. Dopamine analysis

At the end of experimental period, mice were sacrificed by decapitation, Striatum were 28th day and fresh 50 μl (1 $\mu\text{g}/\mu\text{l}$)

striatal lysates (in STEN buffer) were detected with 50 μl primary antibody (1 h) and 100 μl anti-rabbit secondary antibody (30 min) at room temperature according to the manufacturer's protocols (Abnova, Taiwan) for DA [20].

2.4. Estimation of plasma and brain Hcy levels

For analysis of plasma Hcy, the mice were anesthetized with chloral hydrate (350 mg/kg, intraperitoneally) and with the help of fine glass capillary blood was collected from retro-orbital plexus. Plasma was separated out after centrifugation at 1000g for 15 min at 4°C . For estimation of Hcy in nigrostriatum, the right and left nucleus caudatus putamen (NCP; striatum) were dissected out, while the right and left SN were micropunched from 1 mm frozen brain sections, following Palkovits and Brownstein [21]. The right and left hemisphere were processed together for Hcy estimation. The tissues were weighed and sonicated in 5 vol of phosphate buffer saline and stored overnight. After two freeze thaw cycles, the homogenates were centrifuged at 5000g for 5 min and supernatant were analyzed for Hcy content. Total Hcy content was analyzed in plasma or brain homogenates by using Hcy ELISA kit (Abnova, KA1242, Taiwan) as per manufacturer's protocol [22].

2.5. Tyrosine hydroxylase-immunohistochemistry

Mice were anesthetized with chloral hydrate (350 mg/kg; i.p.) and perfused intracardially with phosphate buffer saline (PBS, pH 7.4) followed by 4% paraformaldehyde. Brains were removed and kept in same fixative for overnight, transferred to 30% sucrose and coronal sections (35 μm thickness) passing through SN was taken using Cryotome (0620E Cryostat, Thermo Shandon, UK). The sections were rinsed three times with 0.1 M PBS (pH 7.4), incubated in 3% H_2O_2 in PBS, permeabilized with 0.3% Triton X-100, and blocked with 10% donkey serum containing 0.3% Triton X-100. The sections were incubated with the primary antibody (1:500 dilution; Abcam, UK, ab112) in PBS, containing 2% donkey serum for overnight at 4°C and then incubated with horseradish peroxidase conjugated secondary anti-rabbit antibody (1:1000 dilution; Millipore Co., USA, AP307P) in PBS for 1 h at room temperature. Colour development was performed by incubation the sections in 3, 3-diaminobenzidine liquid substrate system for 3 min and the sections were washed, dehydrated, cleared in xylene, mounted in DPX and photographed (NIKON, ECLIPSE Ci-L, Japan).

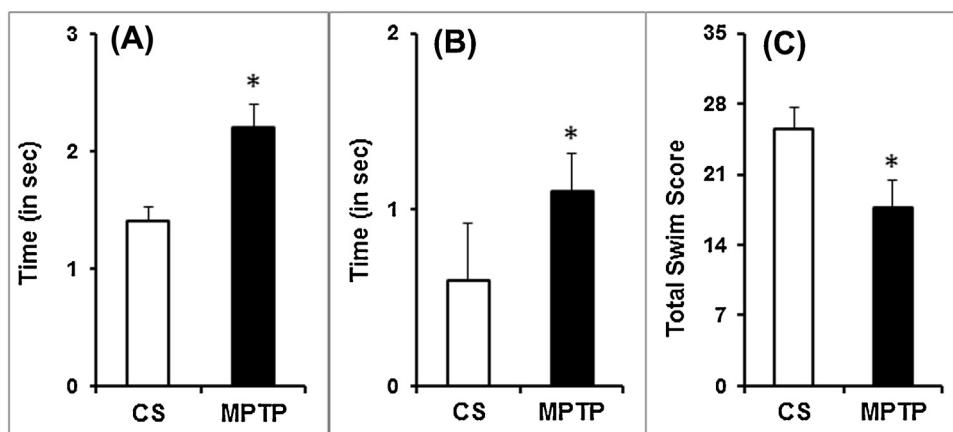


Fig. 1. Effect of MPTP administration on motor behaviour of mice. (A) Akinesia, (B) Catalepsy and (C) Total Swim Score. MPTP treatment in mice, for 5 consecutive days, caused increase in latency (in seconds) in Akinesia and Catalepsy tests, and also decrease in total swim score, which indicate motor behavioural abnormalities. Data represent Mean \pm SEM; $*p \leq 0.05$ as compared to control (C); $n = 6$.

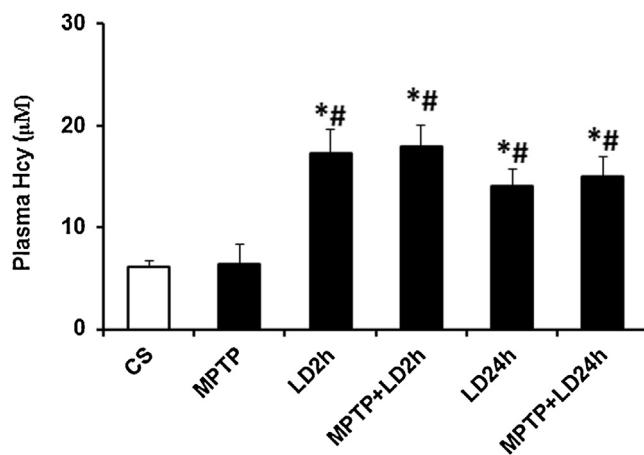


Fig. 2. Effects of chronic L-DOPA administration on plasma homocysteine (Hcy) levels in MPTP mouse model of PD. L-DOPA treatment significantly elevated the level of plasma Hcy in naïve as well as MPTP treated parkinsonian mice at 2 h and 24 h after 28 days of L-DOPA treatment post MPTP treatment, compared to control (CS) and MPTP treated groups. L-DOPA treatment did not exaggerate the Hcy level in the plasma of MPTP treated parkinsonian mice. Results are expressed as μM ; mean \pm SEM. * $p \leq 0.05$ as compared to control; # $p \leq 0.05$ as compared to MPTP alone group. CS = Control; LD = L-DOPA, 2 h and 24 h = time (in hours) after the last dose of L-DOPA.

2.6. Statistics

The group-wise comparison was made using unpaired *t*-test with necessary *post hoc* corrections. One way ANOVA with Dunnett test for *post hoc* analysis was used for comparison of multiple groups with control. Results are given as mean \pm SEM. $p \leq 0.05$ was considered significant.

3. Results

3.1. MPTP-induced PD model

MPTP administration caused significant motor behavioral abnormalities in mice. MPTP treated mice were akinetic as well as cataleptic, and exhibited poor swimming ability as compared to control (Fig. 1). MPTP administration caused 65% depletion in striatal DA contents by 28th day, which were significantly different from the saline treated group (data not shown).

3.2. Plasma Hcy levels after chronic L-DOPA administration in parkinsonian mice

L-DOPA treatment in naïve or MPTP treated mice for 28 days significantly elevates plasma Hcy at 2 h and 24 h after the last dose of the drug (Fig. 2). L-DOPA treatment in naïve mice for 28 days significantly increased the plasma Hcy levels by 3.0- and 2.5-fold at 2 h and 24 h after the last dose of drug respectively (Fig. 2). Interestingly, following 28th dose of L-DOPA in parkinsonian mice, the plasma Hcy level was significantly elevated at 2 h as well as 24 h when compared to the L-DOPA alone treated group as well as control group (Fig. 2).

3.3. Nigrostriatal Hcy levels after chronic L-DOPA administration in parkinsonian mice

The nigrostriatal Hcy level was not significantly affected in MPTP alone treated group. Daily administration of L-DOPA in parkinsonian mice for 28 days significantly elevates Hcy level in SN but not in NCP at either 2 h or 24 h after the last dose of drug compared to L-DOPA group (Fig. 3). In SN, Hcy levels were elevated by more

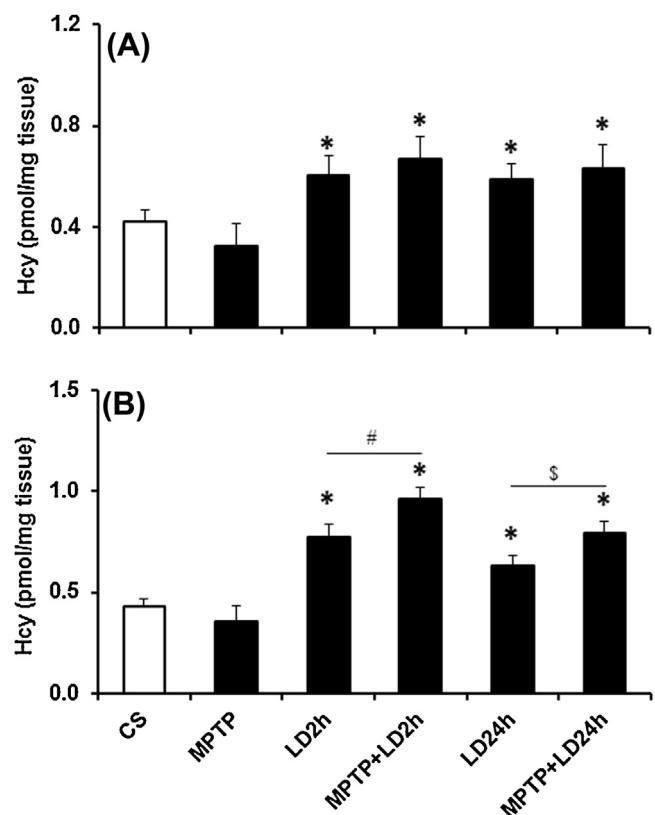


Fig. 3. Effects of chronic L-DOPA administration on (A) Striatal (NCP) and (B) substantia nigral (SN) homocysteine (Hcy) levels in MPTP mouse model of PD. L-DOPA treatment significantly elevated the level of Hcy in the NCP and SN of naïve as well as MPTP treated parkinsonian mice, compared to control (CS). L-DOPA treatment in MPTP-treated parkinsonian mice leads to a significant elevation in the level of Hcy in the SN compared to L-DOPA alone group at both 2 h and 24 h following L-DOPA administration. Results are expressed in picomol/mg brain tissue; Data represent mean \pm SEM. * $p \leq 0.05$ as compared to control, # $p \leq 0.05$ as compared to LD2 h group and \$ $p \leq 0.05$ as compared to LD24 h group (n=6). LD = L-DOPA, 2 h and 24 h = time (in hours) after the last dose of L-DOPA.

than 2-fold at 2 h, while it elevated by more than 1.8-fold at 24 h after the last dose of L-DOPA as compared to control group. However, Hcy level in SN did not differ significantly at 2 h or 24 h when compared with the respective group. Most importantly, treatment of L-DOPA for 28 days in parkinsonian mice significantly elevates Hcy levels in the SN but not in NCP at 2 h and 24 h when compared with respective L-DOPA and MPTP alone treated group as well as control group (Fig. 3).

3.4. TH-positive nigral neurons

MPTP administration caused 47% loss of nigral TH-positive neurons as compared to control (Fig. 4B). In MPTP+L-DOPA group, TH-positive neurons reduced significantly by 51% as compared to control, however, which was not significantly different from MPTP alone treated animals (Fig. 4D).

4. Discussion

The present study reports for the first time a significant elevation of Hcy levels in SN but not in NCP of parkinsonian mice as a result of chronic L-DOPA treatment. The significant elevation of plasma Hcy levels after chronic dosing of L-DOPA in MPTP treated mouse model of PD is consistent with the earlier report [17]. The most important finding of the present study is that SN is susceptible to HHcy induced by L-DOPA in animal models of PD.

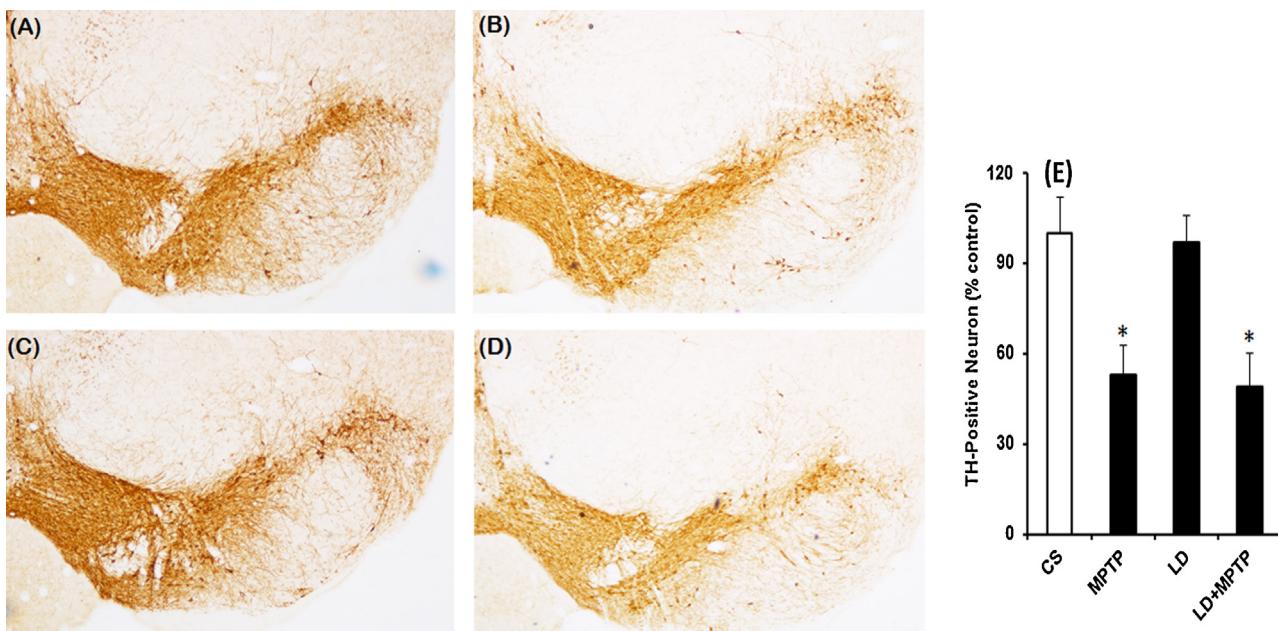


Fig. 4. Effects of chronic L-DOPA administration on tyrosine hydroxylase (TH) positive nigral (SN) neurons in MPTP mouse model of PD. A–D: representative nigral sections of A = Control (CS), B = MPTP treated parkinsonian animals; C = Treated with L-DOPA for 28 days; D = MPTP + L-DOPA group E = TH-positive neuronal count. The number of TH positive neurons in the SN of MPTP treated animals was 51% of CS, while in the MPTP + L-DOPA treated group it was found to be 47%. There is a significant decline in the number of TH positive neurons compared to CS group. Results are expressed as mean \pm SEM. * $p \leq 0.05$ as compared to CS ($n=4$).

From a pilot study (unpublished), we have found that a single dose of L-DOPA in naïve mice leads to a significant increase in plasma Hcy levels from 1 h until 8 h, with highest concentration at 2 h, after which there is a gradual decline in the level of the same. At 24 h the Hcy level of the plasma decreases to that of control. Therefore, we have chosen the two time points (2 h and 24 h) for Hcy estimation both in plasma and brain in the present study. Our result that L-DOPA treatment in parkinsonian mice cause significant elevation of plasma Hcy may be attributed to catechol-O-methyltransferase (COMT) mediated metabolism of the drug [16,23]. The parkinsonian neurotoxin fails to show any significant effect on plasma Hcy levels, which is not consistent with the earlier finding [17]. L-DOPA treatment has recently been reported to increase the Hcy levels in whole brain lysate of parkinsonian mice [17]. In PD patients, increased concentration of Hcy in cerebro spinal fluid was reported [8]. Our result provided the first direct evidence of L-DOPA-induced HHcy in dopamine rich region, particularly in SN, of parkinsonian mice brain. Potentiation of L-DOPA-induced HHcy in SN, but not in NCP, of parkinsonian mice as compared to L-DOPA alone treated mice (Fig. 3B), suggest that SN is more susceptible to HHcy in PD.

Hcy is synthesized mainly in astrocytes, and then transported to neurons [24]. L-DOPA therapy in PD leads to increase in the activity of Catechol-O-methyltransferase, a critical enzyme responsible for the synthesis of Hcy [25,26]. In astrocytes, L-DOPA stimulates COMT and thus leads to enhanced Hcy synthesis and its subsequent export to neuron [17,24]. Thus, it is speculated that the elevation in the level of Hcy in SN, found in the present study, is due to increased COMT activity. In the SN of MPTP + L-DOPA mice, although there is a decrease in the number of dopaminergic neurons compared to MPTP alone group, the same is not significant.

L-DOPA treatment mediated accumulation of Hcy in SN of parkinsonian mice, reported hereby, is of great concern as several studies in cellular as well as animal models have provided the evidences of dopaminergic neurotoxic potency of Hcy [9,12–15]. Moreover, as Hcy is a pro-oxidant molecule [27], increased accumulation of this molecule might alter the redox environment of plasma as well as brain. The potent excitotoxic nature of Hcy

further increases the vulnerability of dopaminergic neurons to L-DOPA therapy [28,29]. However, accumulated Hcy in SN of L-DOPA treated parkinsonian mice did not cause a significant loss of TH-positive neurons (Fig. 4). This indicates that L-DOPA-induced elevation in Hcy level in the SN of parkinsonian mice is not sufficient to cause dopaminergic neurodegeneration. The present study appraised the detrimental effect of L-DOPA in PD in the light of Hcy level in the SN.

Conflict of interest

None declared.

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