

Long-Term L-DOPA Treatment Causes Indiscriminate Increase in Dopamine Levels at the Cost of Serotonin Synthesis in Discrete Brain Regions of Rats

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Abstract (1) The treatment of choice for Parkinson's disease (PD) is 3,4-dihydroxyphenylalanine (L-DOPA) with peripheral decarboxylase inhibitor, but long-term therapy leads to motor and psychiatric complications. In the present study we investigated 5-hydroxytryptamine (5-HT) and dopamine concentrations in serotonergic and dopaminergic nuclei following chronic administration of L-DOPA to find whether the neurotransmitter synthesis in these brain areas are compensated. (2) Rats were administered L-DOPA (250 mg/kg) and carbidopa (25 mg/kg) daily for 59 and 60 days, and killed on the 60th day, respectively at 24 h and 30 min after the last dose. L-DOPA, norepinephrine, 5-HT, 5-hydroxyindoleacetic acid (5-HIAA), dopamine, homovanillic acid (HVA), and 3,4-dihydroxyphenylacetic acid (DOPAC) were measured in striatum, nucleus raphe dorsalis (NRD), nucleus accumbens (NAc), substantia nigra, cerebellum, and cortex employing HPLC-electrochemical procedure. (3) Prolonged treatment of L-DOPA caused depression in the animals as revealed in a forced swim test. Serotonin content was significantly decreased in all brain regions studied 30 min after long-term L-DOPA, except in NAc. The cortex and striatum showed lowered levels of this indoleamine 24 h after 59 doses of L-DOPA. Dopamine, HVA, and DOPAC concentrations were significantly higher in all the regions studied after 30 min, and in the cerebellum after 24 h of L-DOPA. The levels of DOPAC were elevated in all the brain areas studied 24 h after prolonged L-DOPA treatment. (4) The present results suggest that long-term L-DOPA treatment results in significant loss of 5-HT in serotonergic and dopaminergic regions of the brain. Furthermore, while L-DOPA metabolism per se was uninfluenced, dopamine synthesis was severely impaired in all the regions. The imbalance of serotonin and dopamine formation may be the cause of overt cognitive, motor, and psychological functional aberrations seen in parkinsonian patients following prolonged L-DOPA treatment.

Keywords Parkinson's disease · Forced swim test · Dopamine and serotonin synthesis · Motor behavior · Nucleus raphe

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Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by progressive degeneration and loss of nigrostriatal dopaminergic neurons in the midbrain, leading to severe striatal dopamine (DA) depletion culminating in tremor, rigidity, and hypokinesia (Carlsson 1993). Replenishment of striatal DA through the oral administration of 3,4-dihydroxyphenylalanine (L-DOPA), the precursor of DA is the mainstay drug therapy to alleviate the motor symptoms (Hornykiewicz 1998). The enzyme DOPA-decarboxylase or L-aromatic amino acid decarboxylase (AADC) converts not only L-DOPA to DA (Lovenberg et al. 1962; Christenson et al. 1970) but also 5-hydroxytryptophan to 5-hydroxytryptamine (5-HT). This enzyme also converts L-DOPA to dopamine in serotonergic neurons (Arai et al. 1994, 1995, 1996).

The major sites of conversion of exogenous L-DOPA to DA are the dopaminergic nerve terminals. But the mechanism underlying and the site of conversion of L-DOPA to DA in parkinsonian brain, where the neurons or the terminals have been degenerated are not well understood. It has been reported that serotonergic neurons play an important role in this conversion in nigrostriatal lesioned rats (Kannari et al. 2000; Lopez et al. 2001). Arai et al. (1995) have demonstrated that serotonergic fibers of the striatum, raphe nuclei, and neocortex produce DA after intraperitoneal injection of L-DOPA. The existence of AADC in different types of non-monoaminergic striatal neurons (Hefti et al. 1981; Mura et al. 1995) and extra-striatal areas of central nervous system (Jaeger et al. 1983; Brown et al. 1999) has also been reported.

The frequently observed side effects of long-term L-DOPA treatment are the wearing off phenomenon, on-off fluctuations, dyskinesia, and psychosis (Lemke et al. 2004; Hui et al. 2005). Of these, dyskinesia is the most common complication, for which etiology is very complex and unknown, but stimulation of D₁ and D₂ receptors for DA plays a key role (Nutt 1990). In PD, depression may precede the appearance of motor symptoms (Cummings and Masterman 1999). The combined effect of depression and motor symptoms could arise due to long-term effect of L-DOPA therapy. Interactions between nigrostriatal dopaminergic and raphe-striatal serotonergic systems that regulate the activity of striatal neurons (Chesselet and Delfs 1996; Guerra et al. 1998) are important in understanding the pathophysiology of long-term L-DOPA treatment. It has been suggested that chronic use of dopaminergic drugs may lead to psychotic symptoms in PD (Poewe 2003).

In the present study we investigated the effects of prolonged administration of L-DOPA on the steady state level attained after 24 h, and perturbation immediately following its treatment on dopamine and serotonin metabolism in both serotonergic and dopaminergic brain nuclei of normal rats. We hypothesize that any new stable state equilibrium reached by DA and/or 5-HT may lead to motor behavioral abnormalities and psychiatric complications that are exhibited by patients undergoing long-term L-DOPA therapy.

Methods

Animals

Adult male Sprague–Dawley rats (250–300 g) obtained from the institutional animal care facility were housed under standard conditions of temperature ($22 \pm 2^\circ\text{C}$), humidity ($60 \pm 5\%$), and illumination (12-h light–dark cycle). They were provided with food and water ad libitum. All animal experimentation procedures were approved by the Institutional Animal

Ethics Committee and were as per the National Guidelines (CPCSEA) on the Proper Care and Use of Animals in Laboratory Research (Indian National Science Academy 2000).

Drugs and Chemicals

L-DOPA tablets (Syndopa) were purchased from Sun Pharmaceutical Industries Ltd (Baroda, India) containing 10% of carbidopa. Norepinephrine (NE), DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-HIAA, 5-HT, ethylenediaminetetraacetic acid disodium salt (EDTA), and heptane sulfonic acid were purchased from Sigma-Aldrich Co., St. Louis, MO, USA. Acetonitrile was purchased from SISCO Research Laboratories (Mumbai, India).

Experimental Design

First group of animals (Adult male Sprague–Dawley rats, 250–300 g) were treated with L-DOPA (250 mg/kg per day) along with Carbidopa (25 mg/kg per day) by gavage for 59 days and killed on the 60th day (24 h afterwards). The second set of animals was treated with the same dose of L-DOPA with carbidopa for 60 days and neurotransmitters were analyzed 30 min after the last dose of Syndopa. A third group of animals were administered with vehicle. Each group consisted of eight animals.

Daily chronic administration of L-DOPA for 59 days, and analyzing the biogenic amines after 24 h is expected to provide information on whether a steady state level of these neurotransmitters is reached in the brain regions studied or not. Analysis of biogenic amine content 30 min following the 60th dose of L-DOPA is expected to provide information on whether the dopamine precursor conversion in the brain regions is different from the normal.

HPLC Analysis of Biogenic Amines

Brains were removed from the calvarium and the nucleus caudatus putamen (NCP), cortex and cerebellum were dissected out. The substantia nigra (SN), nucleus accumbens (NAc), and nucleus raphe dorsalis (NRD) were micropunched from 1-mm frozen sections (Palkovits and Brownstein 1983). The tissues were processed for the analyses of the biogenic amine neurotransmitters and their metabolites employing an HPLC-electrochemical detection procedure (Mehta et al. 2003; Muralikrishnan and Mohanakumar 1998). The tissues were sonicated in ice-cold 0.1 M HClO₄ containing 0.01% EDTA. The supernatant collected after centrifugation at 10,000 × g for 5 min was injected (10 µl) into the HPLC system (Bioanalytical Systems Inc., West Lafayette, USA) equipped with a pump (PM80), Epsilon amperometric detector (E5) with dual glassy carbon working electrode and Ag/AgCl reference electrode, and a Rheodyne injector. A C18, ion pair, analytical column (4.6 mm × 250 mm; Ultrasphere IP; Beckman, USA), with a particle size of 5 µm and pore of 80 Å was used for separating the amines and their metabolites. The flow rate was 0.7 ml/min and the electrochemical detection was performed at +0.74 V. The composition of the mobile phase was 8.65 mM heptane sulfonic acid, 0.27 mM EDTA, 13% acetonitrile, 0.43% triethylamine, and 0.32% orthophosphoric acid.

Behaviors

Effect of long-term administration of L-DOPA on motor performance was evaluated employing a device to study walking patterns of the animal as described by Klapdor et al. (1997). Walk test was performed following 24 h after 59 days, or 30 min after 60 days of

L-DOPA administration. Walking patterns of rats were analyzed by placing them in an inclining gangway (100 cm long, 12 cm wide with 10 cm high side walls, and a slope of 20°). Rats were pre-trained to walk up the gangway into a dark compartment. Their forelimbs and hindlimbs were painted with two different watercolors and were placed on the gangway. The foot imprints, taken on white papers lined in the gangway, were analyzed as reported earlier (Chandra et al. 2006).

Effect of long-term administration of L-DOPA on depression caused, if any, was evaluated employing a forced swim test as described by Porsolt et al. (1977). Forced swim test was performed 24 h or 30 min following 59 or 60 days daily administration of L-DOPA. Rats were placed in a cylinder (40 cm diameter, × 40 cm height) containing water at 25°C (depth 30 cm). The animals were acclimatized for 15 min, 24 h before the experiment according to the procedure of Porsolt et al. (1977). The swimming was monitored and scored for 5 min by two independent observers. When a rat floated without making any movements for 5 sec the rating was 0, and when it actively swam for 5 sec the score was 1. Total floating time was calculated for the control as well as the treated rats in seconds.

Results

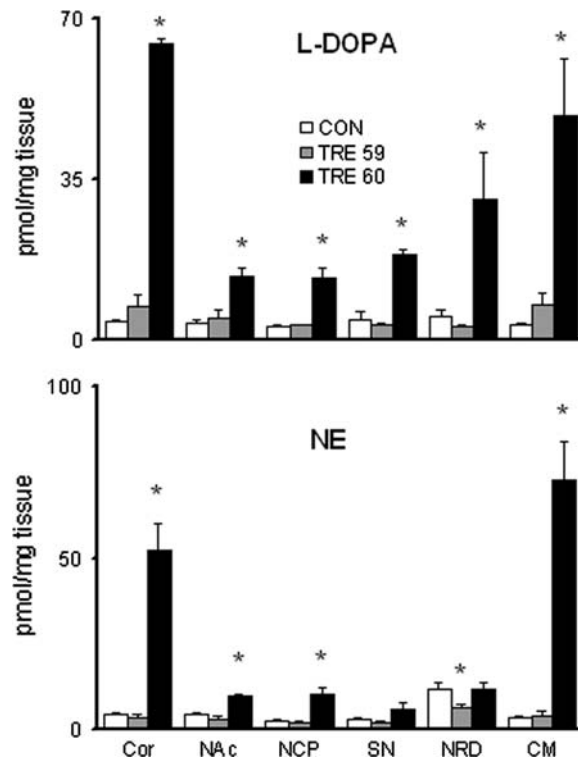
L-DOPA Accumulation is Different in Various Brain Regions

L-DOPA concentrations in different brain regions were found to be comparable to the control value 24 h after chronic administration of L-DOPA. However, the dopamine precursor levels detected in various brain regions were found to be significantly different following 30 min of its administration, as for e.g., while cerebellum and cortex showed 16- and 17-fold increase, NRD, NCP, SN, and NAc depicted respectively 6-, 5-, 4.5-, and 4-fold increase in L-DOPA concentration (Fig. 1; upper panel). Levels of NE was found to be increased in NAc, NCP, cortex, and cerebellum (Fig. 1; lower panel).

Effects of Long-Term Administration of L-DOPA on DA Metabolism in Discrete Brain Regions

In animals administered with L-DOPA for 60 days and analyzed half an hour after the last dose, a significant increase of DA in NCP, SN, NAc, NRD, cortex, and cerebellum was observed (Fig. 2; upper panel). Whereas, in animals administered L-DOPA for 59 days and analyzed on the 60th day, a significant increase of DA was available only in the cortex and cerebellum (Fig. 2). DOPAC was significantly increased in all the regions studied following 30 min or 24 h after prolonged L-DOPA administration (Fig. 2; middle panel). However, HVA was found to be increased in all the brain areas only after 30 min of L-DOPA, but not in animals killed after 24 h (Fig. 2; lower panel). The turnover of DA, as calculated as the ratio of the metabolites to the neurotransmitter [(DOPAC + HVA):DA] has been found to be significantly increased in all the brain regions studied 24 h after the last dose of L-DOPA (Table 1). The increase was 3.4-, 2.6-, 3.6-, 5.1-, 5.3-, and 7.4-folds respectively for the cortex, NAc, NCP, SN, NRD, and cerebellum in animals killed 24 h after 59 days of L-DOPA administration. However the turnover was not significant for cortex and NAc after 30 min of the last dose of L-DOPA. In other brain regions the increase was about 2-fold for all the nuclei, except cerebellum where in augment was 5.7-fold (Table 1).

Fig. 1 Effect of long-term administration of L-DOPA on its and NE contents in the discrete brain regions of rats. Animals were treated with L-DOPA (250 mg/kg, p.o.) along with a peripheral decarboxylase enzyme inhibitor, Carbidopa (25 mg/kg, p.o.) every day for 59 or 60 days, and killed respectively following 24 h (TRE 59) or 30 min (TRE 60) of the last dose of the drugs. Cerebral cortex (Cor), cerebellum (CM), and nucleus caudatus putamen (NCP) were dissected out and nucleus accumbens (NAc), substantia nigra (SN), and nucleus raphe dorsalis (NRD) were micropunched from the brain and processed for the estimation of L-DOPA and norepinephrine (NE) employing a sensitive HPLC-electrochemical detection procedure. Results are Mean \pm S.E.M. * $P \leq 0.05$ as compared to control



Effects of Long-Term Administration of L-DOPA on 5-HT Turnover in Discrete Brain Regions

A significant decrease in 5-HT (Fig. 3; upper panel) and 5-HIAA (Fig. 3; lower panel) levels in all the brain regions, except NAc was seen in the animals analyzed after 60 days of L-DOPA treatment (killed after half an hour of last L-DOPA dose). In animals treated with L-DOPA for 59 days a decrease in 5-HT level was found only in the cortex and NCP after 24 h (Fig. 3). Interestingly 5-HT turnover in any of the brain regions studied was not significantly different from the controls (Table 1).

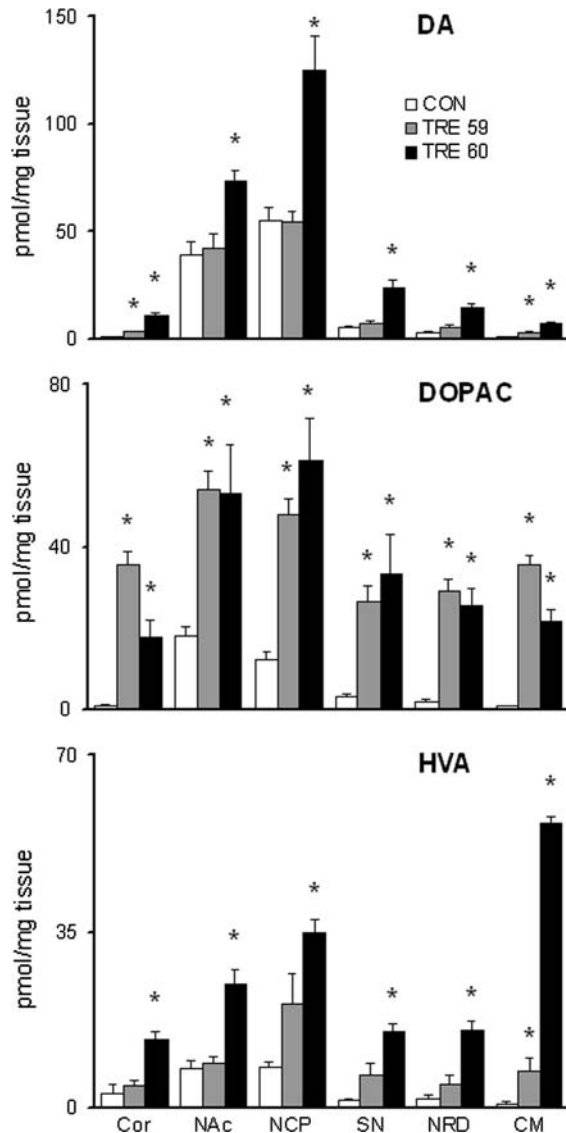
Effect of Prolonged Treatment of L-DOPA on Motor Activity and Depression

No significant changes in the walking patterns (both stride length and stride width) of the animals were observed after prolonged L-DOPA treatment for 59 and 60 days (Table 2). Interestingly, in forced swim test we got a significant increase in the immobility time after chronic L-DOPA treatment in both the experimental groups (Fig. 4).

Discussion

In the present study we have investigated changes in the content of 5-HT, DA and their metabolites in the midbrain raphe nuclei, and the various projection areas in the mesolimbic,

Fig. 2 Effect of long-term administration of L-DOPA on catecholamine contents in the discrete brain regions of rats. Animals were treated with L-DOPA (250 mg/kg, p.o.) along with a peripheral decarboxylase enzyme inhibitor, Carbidopa (25 mg/kg, p.o.) every day for 59 or 60 days, and killed respectively following 24 h (TRE 59) or 30 min (TRE 60) of the last dose of the drugs. Cerebral cortex (Cor), cerebellum (CM), and nucleus caudatus putamen (NCP) were dissected out and nucleus accumbens (NAc), substantia nigra (SN), and nucleus raphe dorsalis (NRD) were micropunched from the brain and processed for the estimation of dopamine (DA); 3,4-dihydroxyphenyl acetic acid (DOPAC) and homovanillic acid (HVA) employing a sensitive HPLC-electrochemical detection procedure. Results are Mean \pm S.E.M. * $P \leq 0.05$ as compared to control



mesocortical, and nigrostriatal system (Steinbusch 1984) following chronic L-DOPA treatment. The salient feature of the study is a steady state, significant decrease in the content of 5-HT in both serotonergic and dopaminergic nuclei following long-term administration of L-DOPA, without a change in its turnover. Another point of interest is the elevated DA metabolism that is persistent in all nuclei studied as indicated by several folds increase in DOPAC level, and in its turnover. The third point of contention is a stably higher level of DA in cortex and cerebellum, two regions where L-DOPA is accumulated maximum 30 min after its administration. The fourth important finding of the present study is the depression caused as a result of prolonged administration of L-DOPA.

Table 1 Dopamine and serotonin turnover in different areas of rat brain after long-term L-DOPA treatment

	Treatment group						
	Cortex	NAc	NCP	SN	NRD	CM	
Dopamine turnover	Control	3.52 ± 1.24	0.65 ± 0.07	0.39 ± 0.04	0.94 ± 0.09	1.24 ± 0.15	1.80 ± 0.32
	Treated 59	12.12 ^{**} ± 1.98	1.68 [*] ± 0.45	1.39 ^{**} ± 0.25	4.84 [*] ± 1.69	6.51 [*] ± 1.88	13.23 ^{***} ± 1.74
	Treated 60	3.00 ± 0.20	1.05 ± 0.18	0.77 ^{***} ± 0.06	2.13 ^{***} ± 0.23	2.59 ^{**} ± 0.30	10.25 ^{**} ± 2.57
Serotonin turnover	Control	2.44 ± 0.49	2.30 ± 0.40	2.00 ± 0.37	1.88 ± 0.37	2.65 ± 0.40	2.22 ± 0.49
	Treated 59	3.37 ± 0.48	2.71 ± 0.53	2.47 ± 0.41	1.98 ± 0.39	2.86 ± 0.67	1.82 ± 0.22
	Treated 60	2.86 ± 0.19	2.09 ± 0.11	2.10 ± 0.10	1.93 ± 0.46	2.33 ± 0.32	2.43 ± 0.71

Animals were treated with Syndopa (L-DOPA; 275 mg/kg, p.o. containing 10% peripheral decarboxylase enzyme inhibitor, Carbidopa) every day for 59 or 60 days, and killed respectively following 24 h or 30 min of the last dose of the drugs. Cortex, cerebellum (CM), and nucleus caudatus putamen (NCP) were dissected out and nucleus accumbens (NAc), substantia nigra (SN), and nucleus raphe dorsalis (NRD) were micropunched and processed for estimation of biogenic amines. The neurotransmitter turnover was calculated as the ratio of the metabolite to the neurotransmitter amine, 5-HT or dopamine. Results are Mean ± S.E.M. * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$ compared to control animals

Fig. 3 Effect of long-term administration of L-DOPA on indoleamine levels in the discrete brain regions of rats. Animals were treated with L-DOPA (250 mg/kg, p.o.) along with a peripheral decarboxylase enzyme inhibitor, Carbidopa (25 mg/kg, p.o.) every day for 59 or 60 days, and killed respectively following 24 h (TRE 59) or 30 min (TRE 60) of the last dose of the drugs. Cerebral cortex (Cor), cerebellum (CM), and nucleus caudatus putamen (NCP) were dissected out and nucleus accumbens (NAc), substantia nigra (SN), and nucleus raphe dorsalis (NRD) were micropunched from the brain and processed for the estimation of serotonin (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) employing a sensitive HPLC-electrochemical detection procedure. Results are Mean \pm S.E.M. * $P \leq 0.05$ as compared to control

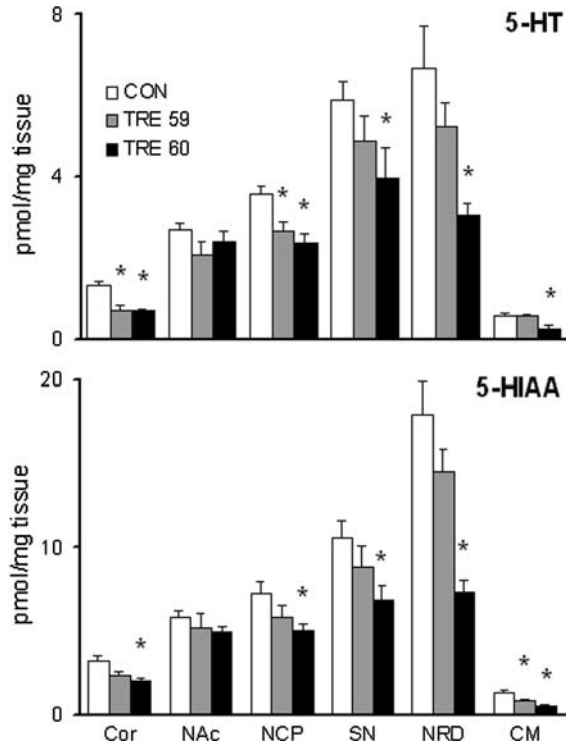
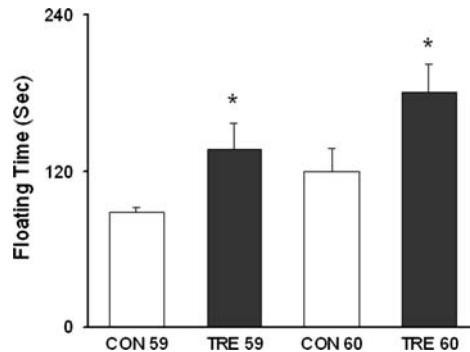


Table 2 Effect of long-term administration of L-DOPA on walking patterns of the rats

	Stride length (cm)	Stride width (cm)
CON	18.05 \pm 0.15	3.99 \pm 0.18
TRE 59	18.88 \pm 0.6	3.88 \pm 0.15
TRE 60	19.15 \pm 0.62	3.98 \pm 0.25

Animals were treated with L-DOPA (250 mg/kg, p.o.) along with a peripheral decarboxylase enzyme inhibitor, Carbidopa (25 mg/kg, p.o.) every day for 59 or 60 days. Walk test was performed following 24 h following 59 (TRE 59) or 30 min after 60 (TRE 60) days treatment of L-DOPA

Fig. 4 Effect of long-term administration of L-DOPA on immobility caused by forced swim. Animals were treated with L-DOPA (250 mg/kg, p.o.) along with a peripheral decarboxylase enzyme inhibitor, Carbidopa (25 mg/kg, p.o.) every day for 59 or 60 days. Forced swim test were performed following 24 h (TRE 59) or 30 min (TRE 60) of the last dose of the drugs. Results are Mean \pm S.E.M. * $P \leq 0.05$ as compared to control animals



A significant decrease (to more than half the control value) in 5-HT and 5-HIAA content in NRD and other serotonergic areas after prolonged L-DOPA administration in the rats may be resulting from competitive L-DOPA uptake into serotonergic neurons in preference to tryptophan by amino acid transporters. L-DOPA in turn is converted to DA in the serotonergic neurons by the enzyme AADC (Hefti et al. 1981; Arai et al. 1995, 1996; Miller and Abercrombie 1999), and when this happens over and over again, it results in stable low levels of 5-HT and its metabolite, 5-HIAA. We have investigated continuous L-DOPA treatment only for a period of 2 months that resulted in half the normal content of 5-HT in NRD. While no motor disability was found in these animals, forced swim test revealed significant incidence of depression in animals treated L-DOPA for 59 and 60 days. This may be caused by the huge loss of 5-HT in major serotonergic nuclei in these rats.

Previous studies have shown that serotonergic fibers of the raphe nuclei can be induced to synthesize DA after L-DOPA treatment in normal rats (Arai et al. 1995), or in mice and rats with extensive dopaminergic denervation in the striatum (Arai et al. 1994; Rozas et al. 1998; Maeda et al. 2005; Kannari et al. 2006). Our results show an increase of DA in both dopaminergic (NCP, SN, NAc) as well as in non-dopaminergic regions (NRD, cortex, and cerebellum) after administration of L-DOPA for 60 days (killed half an hour after the last feed). This increase of DA in dopaminergic regions is obviously due to the transformation of exogenous L-DOPA by AADC. From nucleus raphe many serotonergic projections innervate the basal ganglia, cortex, and cerebellum (Steinbusch 1984; Lavoie and Parent 1990). Therefore, DA levels in NCP, NAc, cortex, and cerebellum may also be attributed to the presence of serotonergic innervations from nucleus raphe. Arai et al. (1995) have shown an increase in DA levels in serotonergic terminal of NCP, NRD, and cortex employing double-labeling immunofluorescence study, whereas we have used a more sensitive HPLC-electrochemical procedure (Muralikrishnan and Mohanakumar 1998) to detect different monoamines. The uptake of DA formed from exogenously administered L-DOPA into the lesioned striatum has been shown to be taking place via serotonergic transporters (Kannari et al. 2006), adding additional dimension to this aspect of DA-5-HT interaction in the striatum. Our study is limited to normal animals, but not in rats with nigrostriatal lesions, and points to robust behavioral and neurochemical changes that occur after prolonged L-DOPA treatment.

Increase of DA in NCP, SN, NRD, and NAc is not stable as seen after 24 h of the last feed of L-DOPA, unlike the increase observed immediately after its administration. Surprisingly DOPAC, the metabolite of DA, shows a steady state increase. This along with the fact that DA turnover has been increased significantly suggests that DA metabolism in the brain nuclei increase after long-term L-DOPA treatment. In PD, the most common side effect of long-term L-DOPA treatment is dyskinesias (abnormal involuntary movements) and motor fluctuations (Chase 1998; Ahlskog and Muentner 2001; Mazzella et al. 2005; Cenci 2007), triggered by transient and large changes in extracellular DA levels (Meissner et al. 2006; Cenci 2007). Here we have demonstrated increases in DA metabolism after long-term L-DOPA treatment (59 days and 60 days) even in normal rat brain, signifying hazards of undergoing extended L-DOPA treatment.

In addition to above, we propose here that long-term L-DOPA treatment in normal rats lead to dysregulated release of DA from the serotonergic projections. This increased levels of DA in the serotonergic terminal acts as “false transmitter” and unlike in dopaminergic neurons, the serotonergic neurons cannot regulate the DA release from these terminals. In our studies, it has been observed that upon L-DOPA administration (killed 24 hrs after the last feed), the level of extra cellular L-DOPA derived DA level is kept in the normal physiological range. However, the metabolite, DOPAC has been shown to be several fold increased and reached a steady state level in all the nuclei studied. Normal release of DA from dopaminergic terminals are

regulated by auto-receptor feedback mechanism and reuptake by DA transporter. However, DA release from serotonergic terminals could not be controlled due to lack of DA autoreceptor regulation, which is similar to what happens in PD where this auto-receptor feedback mechanism is not operative due to lesions in the dopaminergic terminals. This may lead to dysregulated release of L-DOPA derived DA, and probably triggers dyskinesia. Support to this contention is available from studies reporting a reduction of dyskinesia by 5-HT_{1A} or 5-HT_{1B} agonists (Knobelman et al. 2000; Adell et al. 2001; Nicholson and Brotchie 2002; Bishop et al. 2006). The 5-HT agonist may act by decreasing the dysregulated release of DA from the serotonergic terminals.

It is observed that the decrease in serotonin after L-DOPA treatment in animals is not restricted to the serotonergic nuclei, but extends to dopaminergic nuclei too. It is well known that a decrease in 5-HT levels is linked to psychological problems including depression in PD (Kostic et al. 1987; Cummings and Masterman 1999; Leentjens 2004). This aspect also supports the notion of supplementing long-term L-DOPA treatment with serotonergic agents in PD. In a nutshell strategies targeting to increase the serotonin content to normal levels should be considered for the treatment of PD patient undergoing long-term L-DOPA treatment. Our findings are in normal animals treated with L-DOPA for 2 months, and further exhaustive studies are warranted to find the effects of such treatment in animals with nigrostriatal lesions for assessing the situation in PD.

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