Published in final edited form as:

Neurosci Lett. 2015 January 12; 0: 33–37. doi:10.1016/j.neulet.2014.11.023.

Chronic levodopa treatment alters expression and function of dopamine D3 receptor in the MPTP/p mouse model of Parkinson's disease

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Abstract

Chronic treatment with levodopa or antipsychotics results in manifestation of side-effects such as dyskinesia which correlates with changes in expression and function of receptors and signaling proteins. Previous studies have suggested a role for the dopamine D3 receptor in Parkinson's disease (PD) and tardive dyskinesia. Yet the expression and signaling function of D3 receptor in these disorders is not well understood. Here we tested the hypothesis that chronic levodopa treatment alters both expression and function of D3 receptors in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine plus probenecid (MPTP/p) mouse model of PD. *drd3*-EGFP reporter mice were injected biweekly with saline or MPTP and probenecid for a 5-week period. During the last two weeks of the 5-week period, the mice were administered saline or levodopa twice daily. Locomotor activity was measured during the treatment period. D3 receptor expression was determined by western blot analysis. D3 receptor signaling function was determined at tissue and single cell level by measuring the activation of D3 receptor-mitogen activated protein kinase (MAPK) pathway. The *drd3*-EGFP mice administered MPTP/p exhibited akinesia/bradykinesia. Expression of D3 receptor protein in the dorsal striatum specifically increased in the MPTP/p-treated mice administered levodopa. In the dorsal striatum of levodopa and MPTP/p-treated *drd3*-EGFP mice, administration of a D3 receptor-selective dose of agonist, PD128907, failed to activate D3 receptor-MAPK signaling. These results suggest that MPTP-induced lesion and chronic levodopa treatment alters D3 receptor expression and function in the dorsal striatum which could contribute to the development of dyskinesias and other motor side-effects.

Keywords

levodopa-induced dyskinesia; tardive dyskinesia; MPTP; levodopa; *drd3*-EGFP mice; tolerance

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1. Introduction

Among dopamine receptors, the dopamine D3 receptor has the highest affinity for dopamine and dopamine receptor agonists used to treat Parkinson's disease (PD). Changes in dopamine D3 receptor expression have been reported in rat and non-human primate models of PD [1–3] as well as in post-mortem human brain from PD patients [4]. To our knowledge, no published studies have examined changes in D3 receptor protein expression and signaling function in a mouse PD model that exhibits locomotor deficits. Levodopa (L-DOPA) is the gold standard therapy for treating PD; however, most patients develop side-effects including levodopa-induced dyskinesia (LID). Studies in non-human primate models have shown that upregulation of D3 receptor correlates strongly with both LID and tardive dyskinesia [2, 5]. An increase in D3 receptor binding has been reported in MPTP-lesioned C57BL/6 mice administered L-DOPA; however the MPTP treatment protocol in that study did not cause locomotor deficits [6]. We recently showed that overexpression of wild type D3 receptor in dorsal striatum of non-Parkinsonian rats causes LID and promotes vacuous chewing behaviors associated with tardive dyskinesia [7]. Specific knock-down of D3 receptors in the striatum of 6-OHDA lesioned rats has been shown to block the behavioral sensitization elicited by repeated levodopa administration [8]. Changes in D3 receptor sensitization has also been reported in PD and LID [9, 10]. Together the results from these studies suggests that the D3 receptor plays an important role in PD and dyskinesia; unfortunately, the expression and downstream signaling function of D3 receptors in PD and following chronic L-DOPA therapy is poorly understood. To address this, here we tested the hypothesis that chronic L-DOPA treatment changes D3 receptor expression and function in a mouse model of PD that also exhibits locomotor deficits. The hypothesis was tested using the recently characterized *drd3*-EGFP mouse model which express enhanced green fluorescent protein (EGFP) in cells with endogenous D3 receptor expression, facilitating *in vivo* analysis of D3 receptor signaling function [11]. The *drd3*-EGFP mice were rendered Parkinsonian using the well-characterized MPTP + probenecid (MPTP/p) protocol [12–14]. Unlike the traditional MPTP mouse PD model, the MPTP/p mouse PD model exhibits progressive nigro-striatal degeneration and several PD-associated behavioral deficits [14].

2. Experimental Procedures

2.1 Animals

Adult, male 2 to 3 month old *drd3*-EGFP reporter mice weighing about 30 gm were used in this study. The *drd3*-EGFP hemizygous mice (on a mixed Swiss Webster/FVB genetic background) were provided by Drs. Mary Beth Hatten and Nathaniel Heintz (GENSAT program, Rockefeller University) and a breeding colony was established at Rutgers University-New Jersey Medical School. The *drd3*-EGFP reporter mice have been previously characterized by us in detail [11, 15]. Mice were group-housed (4 or less per cage) with littermates in a temperature and humidity controlled environment, with a 12 hr light (on at 7 A.M.): dark (off at 7 P.M.) cycle. All experiments were performed in the light phase. Mice were provided access to standard rodent chow and water *ad libitum*. Chow pellets and HydroGel™ (ClearH2O, Portland, ME, USA) were placed on the floor of cages with MPTP/p-treated mice that exhibited severe motor deficits. The mice in various groups did

not exhibit significant difference in body weight during the treatment period. All procedures were approved by the IACUC committee at Rutgers-New Jersey Medical School.

2.2 Drugs

1-methyl-4-phenyl,1,2,3,6-tetrahydropyridine (MPTP; Sigma, St. Louis, MO, USA) and probenecid (Sigma, St. Louis, MO, USA) were dissolved to desired concentration in saline and injected intraperitoneally (i.p.) at a dose of 25 mg/kg and 250 mg/kg, respectively. Probenecid stock solution (12.5 mg/mL) was prepared by dissolving the probenecid in 0.1 N NaOH and titrating to pH 7.4 using 1 M Tris-HCl (pH 6.8). Levodopa methyl ester (L-DOPA; Sigma) and benserazide hydrochloride (Sigma) were dissolved in saline and injected i.p. at a dose of 25 mg/kg each. The drugs were injected according the scheme shown in Figure 1A. To test D3R signaling function, R-(+)-trans-3,4,4a,10b-tetrahydro-4-propyl-2 H, 5 H-(1)benzopyrano[4,3-b]-1,4-oxazine-9-ol (PD128907; Tocris, Minneapolis, MN, USA) was dissolved in saline and injected subcutaneously (s.c.) at a dose of 0.05 mg/kg.

2.3 Locomotor activity measurement

Horizontal locomotor activity was measured using the open field photobeam activity system (PAS; SD Instruments, San Diego, CA, USA) and the data collected and analyzed as previously reported [11]. Locomotor activity was assessed approximately twelve hours after one, six and ten injections of MPTP/p.

2.4 Western blotting

Tissue harvest, western blotting and the conditions for detecting pERK, tERK, and D3 receptors have been described previously [11, 16]. D3 receptor antibody was validated prior to use (Supplementary Figure 1). Phosphorylated ERK protein (pERK) levels were normalized to their corresponding total protein levels (tERK). D3 receptor levels were normalized to total protein loaded in each lane which was determined by staining with the blot with amido black stain as described previously [11, 16]. Experiments were repeated at least three independent times with comparable results.

2.5 Immunohistochemistry

Protocols used for immunohistochemistry and staining of phosphorylated ERK in the striatum of *drd3-EGFP* mice have been described recently in detail [11]. The number of cells co-staining for EGFP and phosphorylated ERK was quantitated as described previously [11].

2.6 Statistical analyses

All statistical analysis were performed with the SigmaPlot® 11 software (SPSS Inc.). Data were considered statistically significant when the probability value (P) was less than 0.05.

3. Results

3.1 *drd3*-EGFP mice chronically injected with MPTP/p exhibit motor deficits

The chronic MPTP/p protocol used in this study has been previously described and validated for progressive PD pathogenesis including severity of lesion as well as motor and olfactory deficits in C57BL/6 mice [12–14, 17]. To determine if the novel *drd3*-EGFP mice, with a mixed Swiss Webster/FVB genetic background, develops motor deficits following the MPTP/p treatment protocol, we utilized the treatment schedule shown in Figure 1A. Adult male *drd3*-EGFP mice were injected twice a week for 5 weeks with 25 mg/kg MPTP plus 250 mg/kg probenecid (MPTP/p). Figure 1B illustrates that a single injection of MPTP/p produces a significant decrease in locomotor activity compared to a saline control. The results in Figure 1B also demonstrate that by the third week of biweekly MPTP/p injections, the locomotor deficit is severe and does not differ from the deficit seen on the last day of injections. Supplementary video 1 and video 2 shows the relative locomotor activities of saline and MPTP/p treated mice after 10 injections, respectively. The MPTP/p-treated mouse in Supplementary video 2 clearly exhibits akinesia/bradykinesia. These results suggest that, similar to the C57BL/6 strain, the *drd3*-EGFP mouse strain with the Swiss Webster/FVB background exhibits severe motor deficits following chronic MPTP/p treatment.

3.2 Increase in D3 receptor protein expression in dorsal striatum of MPTP/p-lesioned *drd3*-EGFP mice chronically administered L-DOPA

To determine the level of D3 receptor protein expression in the dorsal striatum of control and MPTP/p-lesioned *drd3*-EGFP mice, we harvested brain tissue ~18 hours after the last injection of the 5-week treatment period from mice in the four treatment groups and performed western blot analysis. The results in Figure 2 show that compared to the three other treatment groups there was a significant increase in D3 receptor protein levels in the treatment group in which MPTP/p-lesioned mice were administered daily L-DOPA. The specific increase in D3 receptor expression in the MPTP/p-lesioned L-DOPA-treated mice is consistent with previous results from rodents and non-human primates [1–3, 6].

3.3 Loss of D3 receptor signaling function in dorsal striatum of MPTP/p-lesioned and L-DOPA-treated *drd3*-EGFP mice

We have recently shown that 0.05 mg/kg PD128907 selectively activates the D3 receptor-MAPK signaling pathway and increases pERK in the dorsal striatum of the *drd3*-EGFP mice [11]. To determine D3 receptor signaling function in control and MPTP/p-lesioned mice, we administered a D3 receptor-selective dose of agonist, PD128907, to mice in the four treatment groups and measured the change in pERK levels in the dorsal striatum. Approximately 18 hours after the last injection of the 5-week treatment period, mice in the four treatment groups were administered saline or 0.05 mg/kg PD128907 and dorsal striatum harvested 10 minutes later for western blot analysis. Consistent with our previous results [11], 10 minutes after the administration of 0.05 mg/kg PD128907, there is a significant increase in pERK in vehicle (saline)-treated *drd3*-EGFP mice; however, no PD128907-induced increase in pERK was observed in L-DOPA-treated mice, MPTP/p-lesioned mice or MPTP/p-lesioned mice treated with L-DOPA (Fig. 3).

Interestingly, under these experimental conditions, the mice in the MPTP/p-treated group showed a significant decrease in basal pERK levels which was reversed when the MPTP/p-treated mice were chronically administered L-DOPA during the last two weeks of the 5-week period. As previous studies have shown that MAPK signaling pathway and pERK levels are also modulated by dopamine D1 receptors, DARPP-32 and striatal enriched phosphatase (STEP), we measured the basal levels and activity of these proteins in the dorsal striatum of MPTP/p-treated mice and MPTP/p-treated mice chronically administered L-DOPA [18, 19]. The data in Supplementary Figure 2 shows that there is no difference in D1 receptors, phospho-DARPP-32 or non-phosphorylated STEP protein levels between the two treatment groups.

We next determined if the loss of D3 receptor function observed in total striatal tissue is also observed specifically in the D3 receptor-expressing cells of the dorsal striatum. As the *drd3*-EGFP mice express the fluorescent EGFP in cells that express endogenous D3 receptor mRNA, we determined the levels of PD128907-induced pERK in the fluorescent D3 receptor-expressing cells of the dorsal striatum of the *drd3*-EGFP mice from each of the four treatment groups. Brain tissue was harvested for immunohistochemistry, 10 minutes after administering saline or 0.05 mg/kg PD128907. Consistent with the western blot results in Figure 3, the results in Figure 4 show that PD128907 significantly increases the percentage of cells co-expressing pERK and D3 receptors, in saline-treated mice (also see Supplementary Figure 3). However, PD128907 did not increase the percentage of cells co-expressing D3 receptor and pERK in L-DOPA administered mice, MPTP/p-lesioned mice or MPTP/p-lesioned mice administered L-DOPA (Fig. 4 and Supplementary Fig. 3). Taken together the results in Figure 3 and 4 suggest that both chronic L-DOPA treatment and MPTP/p-induced lesion causes a loss of D3 receptor-MAPK signaling function in the *drd3*-EGFP mice.

4. Discussion

In this paper we show, for the first time, that chronic L-DOPA treatment of MPTP/p-lesioned mice results in increase of D3 receptor expression but loss of D3 receptor-MAPK signaling function in the dorsal striatum of *drd3*-EGFP mice. Analysis of the immunohistochemistry images (Supplementary Figure 3) suggest that relatively few cells in the dorsal striatum express D3 receptors and the number of cells expressing EGFP in the dorsal striatum does not change across the four treatment groups. We also observed EGFP expression in the fibers innervating dorsal striatum, which suggest that D3 receptor expression likely occurs in afferents innervating dorsal striatum. It has been shown that D3 receptors are expressed in asymmetric glutamatergic synapses and potentially modulates release of glutamate from cortico-striatal terminals [20].

Interestingly, despite the increase in D3 receptor protein expression in the dorsal striatum of the L-DOPA-treated, MPTP/p-lesioned mice, there is a loss of D3 receptor-MAPK signaling function both in the D3 receptor-expressing cells and the fibers innervating the dorsal striatum. This loss of D3 receptor-MAPK signaling is also observed in non-lesioned L-DOPA-treated mice and untreated MPTP/p-lesioned mice. Previous studies using the MPTP/p mouse model have shown that while MPTP/p lesion decreases extracellular striatal

glutamate levels, L-DOPA treatment of MPTP/p-lesioned mice restores the glutamate levels and activates NMDA receptors [17, 21]. It has been shown that the increase in intracellular calcium via activation of NMDA receptors promotes D3 receptor-calcium/calmodulin-dependent protein kinase II (CAMKII) interactions which downregulates D3 receptor function [22]. The NMDA receptor-CAMKII-mediated decrease in D3 function provide a potential explanation for the loss of D3 receptor function that we observed in the L-DOPA-treated, MPTP/p-lesioned *drd3*-EGFP mice. Interestingly, it has also been shown that dopamine depletion in a rat PD model constitutively increases the phosphorylation of CAMKII (Thr 286) [23]; which could lead to binding of CAMKII to D3 receptor and inhibition of D3 receptor function [22]. This mechanism could explain the loss of D3 receptor function in untreated MPTP/p-lesioned mice. The aberrant levodopa-induced increase in D3 receptor expression combined with the loss of D3 receptor signaling function in the MPTP/p-lesioned mice reported in this study might contribute to dysregulated glutamate and dopamine release resulting in the development of dyskinesia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the New Jersey Health Foundation and the F. M. Kirby Foundation to EVK. SRC was supported by a NIH T32 predoctoral training grant (NS 51157-5) and a Summer Fellowship Award from the Parkinson's Disease Foundation.

Abbreviations

PD	Parkinson's disease
MPTP/p	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine plus probenecid
L-DOPA	levodopa
LID	levodopa-induced dyskinesia
MAPK	Mitogen-activated protein kinase
pERK	phosphorylated extracellular signal-regulated kinase
tERK	total extracellular signal-regulated kinases
PAS	photobeam-activated system
CAMKII	calcium/calmodulin-dependent protein kinase II
EGFP	enhanced green fluorescent protein
i.p	intraperitoneal
s.c	subcutaneous

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Highlights

- Chronic MPTP/p treatment causes akinesia/bradykinesia in *drd3*-EGFP mice.
- L-DOPA-treated MPTP/p-lesioned mice show increased striatal D3 receptor expression.
- Striatal D3 receptor function is repressed after L-DOPA treatment or MPTP/p lesion.
- MPTP/p-lesioned mice show decreased basal phospho-ERK levels in the striatum.
- L-DOPA increases basal phospho-ERK levels in the striatum of MPTP/p-lesioned mice.

Figure 1.

Chronic treatment with MPTP and probenecid (MPTP/p) induce motor deficits in *drd3-EGFP* mice. **(A)** *drd3-EGFP* mice were divided into 4 groups ($n=12$ mice per group) that included saline, L-DOPA, MPTP/p and MPTP/p + L-DOPA. Mice were administered 10 injections (black arrows) of saline or MPTP/p (25 mg/kg MPTP + 250 mg/kg probenecid) for 5 weeks. During the last two weeks of the 5-week period (horizontal dashed arrow) mice were injected twice daily with saline or 25mg/kg L-DOPA + 25mg/kg benserazide. Black triangles indicate when locomotor activity was measured in the MPTP/p group. Black diamond indicates when video of motor deficit was recorded in the MPTP/p group. Approximately 18 hours after last injection of the 5-week treatment period, the mice were sacrificed and tissue harvested for western blotting (WB) and immunohistochemistry (IHC). **(B)** Locomotor activity was recorded in mice from the saline- and MPTP/p-treated groups during the 5-week treatment as the total distance traveled during a 60 minute period in an open field arena. The locomotor data for saline group was from mice administered 10 saline injections during the 5-week period. Error bars represent $\pm$ SEM. *, $P < 0.01$, statistically significant decrease in locomotor activity was observed between the MPTP/p- and saline-treated groups, as well as between mice administered one MPTP/p injection and six or ten MPTP/p injections, One Way rm-ANOVA, post-hoc Holm's-Sidak test.

Figure 2.

Representative western blot (A) and cumulative data (B) showing levels of D3 receptor protein in dorsal striatum of *drd3*-EGFP mice administered only saline (during the 5-week injection period), L-DOPA (5-week bi-weekly saline injection plus L-DOPA twice daily for last 2 weeks), MPTP/p (5-week bi-weekly MPTP/p injection plus twice daily saline injection for last 2 weeks) and MPTP/p+L-DOPA (5-week bi-weekly MPTP/p injection plus twice daily L-DOPA for last 2 weeks). D3 receptor levels were normalized to total protein loaded in each lane which was detected using the amido black stain. N=5 mice per group. Error bars represent $\pm$ SEM. *, $P < 0.05$, statistically significant increase in D3 receptor protein levels was observed only in the MPTP/p+L-DOPA treatment group, One Way ANOVA, post-hoc Dunn's test.

Figure 3.

Loss of striatal D3 receptor-MAPK signaling function in *drd3*-EGFP mice chronically treated with L-DOPA and/or MPTP/p. Representative western blot (**A**) and cumulative data (**B**) showing levels of pERK and tERK in dorsal striatum of *drd3*-EGFP mice 10 minutes after administration of saline (black bar) or D3 receptor-selective dose of PD128907 (0.05 mg/kg; white bar) to each of the four treatment groups. Values represent ratios of pERK/total ERK protein levels normalized to saline controls. N=6 mice per group. Error bars represent $\pm$ SEM. *, $P < 0.05$, statistically significant PD128907-induced increase in pERK levels were observed only in the saline treatment group; #, $P < 0.01$, statistically significant decrease in basal pERK levels were observed in the MPTP/p-lesioned group, compared to all other groups, One Way ANOVA, post-hoc Dunn's test.

Figure 4.

Loss of D3 receptor-MAPK signaling function in the D3 receptor-expressing cells in the dorsal striatum of *drd3*-EGFP mice chronically treated with L-DOPA and/or MPTP/p. Percent of cells co-expressing pERK and D3 receptor mRNA in the dorsal striatum of *drd3*-EGFP mice from each of the four treatment groups treated with either saline (black bar) or PD128907 (0.05 mg/kg, white bar) were determined by counting the number of green fluorescent, D3 receptor-expressing cells with and without pERK co-staining. Data were obtained by analyzing IHC images from 5 independent coronal sections of dorsal striatum from three different mice in each treatment group. Error bars represent $\pm$ SEM. *, $P < 0.05$, statistically significant PD128907-induced increase in percentage of cells co-expressing EGFP and pERK was observed only in the saline treatment group, One Way ANOVA, post-hoc Dunn's test.