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Research report

Absence of inclusion body formation in the MPTP mouse model of Parkinson's disease

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Abstract

Formation of α-synuclein aggregation and Lewy bodies (LBs) are hallmarks of Parkinson's disease (PD) and other related diseases. The dopaminergic neurotoxin, MPTP, replicates many of the pathological signs and motoric features of PD in primates and rodents by selective destruction of dopamine (DA) neurons of the substantia nigra. In this study, groups of adult wild-type C57BL6 mice were treated with MPTP either acutely (20 mg/kg, every 2 h × 4 for 1 day), semi-chronically (30 mg/kg/day for 5 days), or chronically (25 mg/kg MPTP with 250 mg/kg probenecid 2 times/week for 5 weeks). Mice brains were collected and processed at various time points for immunohistochemistry and HPLC assays. Our data showed that although there is a significant decrease in DA content and its metabolites and tyrosine hydroxylase immunoreactivity, there is no inclusion body formation following the various MPTP treatment regimens.

Theme: Neurobiology of disease
Topic: Parkinson's disease

Keywords: MPTP; Parkinson's disease; Animal model; α-synuclein; Ubiquitin; Dopamine; Dopac; HVA; Tyrosine hydroxylase

1. Introduction

Aggregation of α-synuclein and Lewy body (LB) formation are hallmarks of Parkinson's disease (PD) and other related diseases [1,30,42]. PD is a late-onset progressive motor disease marked by selective degeneration of dopamine (DA) neurons of the substantia nigra (SN) [13,22] and formation of fibrillar cytoplasmic inclusions known as Lewy bodies (LBs) [30]. PD behavioral manifestations are characterized by tremor, bradykinesia, rigidity, and postural instability. Although the exact etiology of PD is unknown, it is thought that impaired energy metabolism and factors leading to increased oxidative stress may be involved in DA neuronal cell death [21].

LBs predominantly contain a dense protein aggregation of fibrillar forms of α-synuclein that stain for ubiquitin [4,36,42]. The α-synuclein aggregation is thought to be the precursor of LBs. The molecular mechanism underlying α-synuclein aggregation in vivo remains unknown.

Some of the neuropathology of PD can be elicited by selective destruction of DA neurons of SN following administration of the neurotoxin, 1-methyl-4-penyl-1,2,3,6-tetrahydropyridine (MPTP) in primates and rodent animal models [5,26,35]. MPTP replicates parkinsonian motor signs in human and nonhuman primates [2,6,12,14,17,23–25,33,38].

MPTP exerts its neurotoxicity through its active metabolite, 1-methyl-4-phenyl pyridium (MPP^+) [29]; through monoamine oxidase B metabolism of MPTP to MPP^+ [20]. MPTP neurotoxicity causes selective DA neuron degeneration by concentrating MPP^+ in DA neurons via the DA transporter and selectively inhibiting mitochondrial complex 1. One potential mechanism of the action of MPTP on DA

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neuron toxicity is inhibition of mitochondrial complex 1 and the formation of the superoxide anion coupled with generation of both neuronal and microglial-derived NO to form peroxynitrite that are thought to damage DA neurons oxidatively through DNA damage and activation of poly (ADP-ribose) polymerase [19,27,28,37]. MPTP kills DA neurons through additional pathways including caspase-dependent and -independent pathways and induction of local inflammatory pathways in the substantia nigra [9].

Due to its specific toxicity to the DA neurons of the SN where PD pathology is detected, MPTP is widely used to induce an animal model of PD [2,6,24]. There are several other animal models of PD (see Refs. [9,11] for review). Overexpression of α-synuclein in *Drosophila*, rodents, and non-human primates leads to α-synuclein inclusion formation [9,11]. Chronic proteasomal inhibition has been reported to induce α-synuclein inclusion formation and selective loss of DA neurons in rats [31]. A relatively invasive method via jugular vein cannula implantation filled with a naturally occurring pesticide, rotenone, kills DA neurons with α-synuclein aggregation and behavioral characteristics of PD [3].

The MPTP mouse model has led to tremendous insight into the molecular mechanisms by which DA neurons in the SN die [9]. Since α-synuclein aggregation is promoted, in part, through oxidative stress, and mice lacking the gene for α-synuclein [10] are resistant to the toxic effects of MPTP, we wondered whether α-synuclein aggregates and forms inclusions following MPTP intoxication.

In this study, groups of wild-type C57BL6 mice were treated with MPTP acutely, semi-chronically, or chronically (Table 1). Mice brains were processed for TH, α-synuclein and ubiquitin immunohistochemistry and HPLC analysis performed for DA and its metabolites. Our data show that although there were significant decreases in DA contents and its metabolites and TH immunoreactivity, there was no inclusion body formation following the various MPTP treatment regimens.

2. Materials and methods

2.1. Animals

All experiments with mice conformed to approved guidelines by the Institutional Animal Care Committee. Wild-type male C57BL6 mice (Charles River) (60 to 90 days old) were used. Animals were kept in groups of 4 to 5 per cage in a temperature-controlled room with a 12-h light/dark cycle and free access to food and water.

Table 1
MPTP (MPTP-HCl) injection methods

Injection types	Dosage	Tissue collection
Acute	24 mg/kg × 4 for 1 day	7 days later
Semi-chronic	36 mg/kg/day × 5 days	3 weeks later
Chronic	30 mg/kg/day × 2 times/week × 5 weeks	>5 weeks later; up to 6 months

2.2. Human tissue

Human brain tissue was obtained through the brain donation program of the Morris K. Udall Parkinson's Disease Research Center at Johns Hopkins Medical Institutions (JHMI) according to HIPAA regulations. This research proposal involves anonymous autopsy material that lacks identifiers of gender, race, or ethnicity. The JHMI Joint Committee on Clinical Investigations decided that the studies in this proposal are exempt from Human Subjects Approval because of Federal Register 46.101 exemption number 4. Tissue from control brains and PD brains were utilized for immunohistochemistry for ubiquitin and α-synuclein.

2.3. MPTP treatment

Animals were treated with MPTP with various regimens: acutely (20 mg/kg free base × 4, i.p.), semi-chronically (30 mg/kg/day free base × 5 days, i.p.) or chronically (25 mg/kg free base with 250 mg/kg probenecid 2 time/week × 5 weeks; sc) (Table 1). For the chronic MPTP treatment, a probenecid (Sigma Chemical, St. Louis, MO, USA; dissolved in 100% DMSO) injection (i.p.) was given 30 min prior to each MPTP injection. MPTP is rapidly metabolized and excreted as MPTP N-oxide within the first 3 h of administration. Probenecid administration inhibits urinal excretion of MPTP and its metabolite, therefore, prolonging retention of MPTP. It has been reported that probenecid alone does not affect DA and its metabolites [26].

2.4. Immunohistochemistry

Brains of MPTP-treated mice were collected at various time points (Table 1), and brains were sectioned (40 μm thickness) following perfusion and fixation. Immunoreactivity to tyrosine hydroxylase (TH), α-synuclein, and ubiquitin were examined by immunohistochemistry. All brain sections were first treated with 1% sodium borohydride (Sigma Chemical, St. Louis, MO, USA) for 30 min at room temperature (RT) followed by 5 times 10 min wash in 1× tris-buffer saline (TBS) at RT. Brain sections were then permeabilized in 0.4% Triton X-100 (Sigma Chemical, St. Louis, MO, USA) at 4 °C for 30 min, then blocked in 4% bovine serum albumin (Sigma Chemical, St. Louis, MO, USA), 0.2% Triton X-100, and 0.02% sodium azide (NaN_3, Sigma Chemical, St. Louis, MO, USA) at 4 °C for 30 min. Primary antibodies, rabbit-anti-mouse α-synuclein (1:4000; Chemicon International Inc., Temecula, CA, USA), mouse-anti-ubiquitin (1:1000; Research Diagnostics Inc., Flanders, NJ, USA), and mouse-anti-TH (1:2000; Incstar) were diluted in 2% BSA, 0.2% Triton X-100, and 0.02% NaN_3

and incubated at 4 °C for >24 h. Secondary antibodies, biotinylated goat-anti-rabbit, or goat-anti-mouse antibodies (1:1000; Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA) were incubated for 45 min at RT. ABC VECTOR immunodetection kits and DAB were used to detect specific staining for α-synuclein, ubiquitin, and TH.

2.5. HPLC analysis

Some of the MPTP treated brains were used for HPLC assays of DA and its metabolites (DOPAC and HVA) with electrochemical detection as described previously [37]. Briefly, samples were sonicated in 200 μl of 0.1M of perchloric acid containing 0.01% ascorbic acid and 25 μg/ml of 3,4-dihydrobenzylamine (Sigma) as an internal standard. Following centrifugation (15,000 × *g*, 15 min, 4 °C), the supernatant was filtered through 0.45 μm pore filters. 20 μl of the filtered sample was injected onto a C18-reverse phase RP-80 catecholamine column (ESA, Bedford, MA). The mobile phase consisted of a solution containing 0.15 M monochloroacetic acid, 200 mg/L sodium octyl sulfate, 0.1 mM EDTA, 4% acetonitrile (filtered) and 2.5% tetrahydrofuron. The flow rate was set at 1.0 ml/min. Peaks were detected by a Coulochem 5100 A detector (E1 = −0.04V, E2 = +0.35V) (ESA Inc., Chelmsford, MA, USA).

3. Results

3.1. DA contents and its metabolites in striatum

DA levels and its metabolites in striatum following MPTP treatments were assayed by HPLC (Fig. 1). All three MPTP treatments reduce striatal DA content significantly compared to control (CTL) animals (Fig. 1A) confirming effectiveness of MPTP delivery. In the acute (Fig. 1B) and semi-chronic paradigms (Fig. 1C), both striatal DA and DOPAC are significantly decreased. In the chronic paradigm (Fig. 1D), only DA is significantly decreased. The observation of no significant changes of DOPAC in the chronic paradigm and HVA in all three paradigms might be due to the fact that the concentration and/or the signal/noise ratio of these dopamine metabolites are too low to be precisely determined by our current HPLC method. It could also be a result of different responses of dopaminergic neurons to different MPTP treatments. However, the

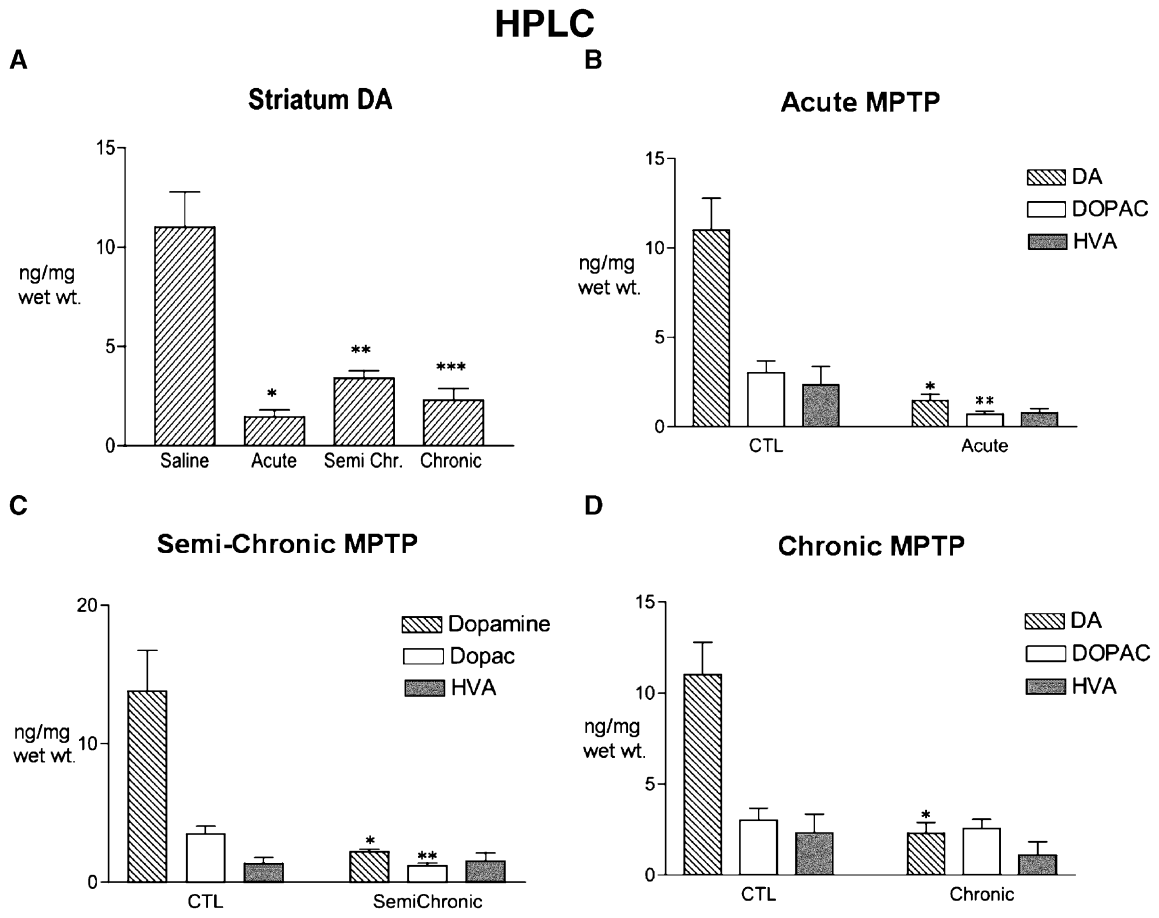

Fig. 1. HPLC analysis of DA and its metabolites in striatum. (A) Comparison of striatal DA with the three different MPTP regimens *$P < 0.009$, $t = 4.11$, **$P < 0.01$, $t = 3.96$, ***$P < 0.007$, $t = 3.94$. DA metabolites with (B) acute *$P < 0.009$, $t = 4.11$, **$P < 0.006$, $t = 4.48$, (C) semi-chronic *$P < 0.01$, $t = 3.96$, **$P < 0.009$, $t = 3.81$ and (D) chronic MPTP treatments *$P < 0.007$, $t = 3.94$.

significant decrease of DA indicates that dopaminergic neurons are all intoxicated by MPTP in these three paradigms.

3.2. TH immunohistochemistry

Immunohistochemistry of mice brains reveals a significant decrease of TH-positive neurons the SN pars compacta following acute, semi-chronic and chronic MPTP treatments (Fig. 2). The decline of TH immunoreactivity within the SN pars compacta indicates that MPTP is neurotoxic to dopaminergic neurons.

3.3. Ubiquitin and α-synuclein immunohistochemistry

Although both HPLC and TH immunohistochemistry demonstrated decreased DA content and the loss of DA neurons, respectively, in all three treatment paradigms, there is no accumulation of ubiquitin-positive (Figs. 3A–D) or α-synuclein-positive inclusions (Figs. 3E–H) within the substantia nigra pars compacta.

We also did not observe Lewy body or Lewy body-like protein aggregates in TH-positive neurons of SN pars compacta from MPTP-treated mouse brains using fluorescence double staining and confocal microscopic examinations with TH and α-synuclein or ubiquitin (data not shown). Human SN tissue from a patient with Parkinson's disease to serve as a positive control (Figs. 3J and L) and control tissue from a subject without neurodegenerative disease (Figs. 3I and K) were examined for immunoreactivity to ubiquitin (Figs. 3I and J) and α-synuclein (Figs. 3K and L). As expected, there are inclusions with positive immunoreactivity to ubiquitin and α-synuclein in the SN from PD patients while no immunoreactivity is observed in control SN. These results taken together suggest that α-synuclein fails to accumulate following MPTP intoxication in mice.

4. Discussion

The major finding of this study is that MPTP fails to induce the formation of α-synuclein inclusions or ubiquitin immunoreactivity in three different MPTP intoxication paradigms. Even though all three paradigms induce a significant decrease of DA and it metabolites and the loss of DA neurons, surprisingly, there is no inclusion formation.

MPTP administration upregulates α-synuclein mRNA and protein expression in DA neurons of the SN [40]. α-Synuclein and its related family members are abundant neuronal cytosolic proteins enriched at presynaptic terminals and are thought to be involved in synaptic function and plasticity [7]. α-Synuclein is a major component of LB and neurites, and it is abundant in pale bodies that are believed to be the precursor of LB [39]. It is still unknown, however, how α-synuclein contributes to the cellular and biochemical mechanisms of PD, and its normal functions and biochemical properties are also not well understood. Both wild-type and mutant α-synuclein can self-aggregate and assemble into fibrils that resemble the ultrastructural elements of LB [8]. α-Synuclein can also bind to a variety of proteins that could contribute to possible toxic properties [15,16,41]. α-Synuclein may be selectively and specifically nitrated, and it may link oxidative and nitrative damage to the onset and progression of neurodegenerative synucleinopathy lesions [18]. Moreover, oxidative stress can lead to

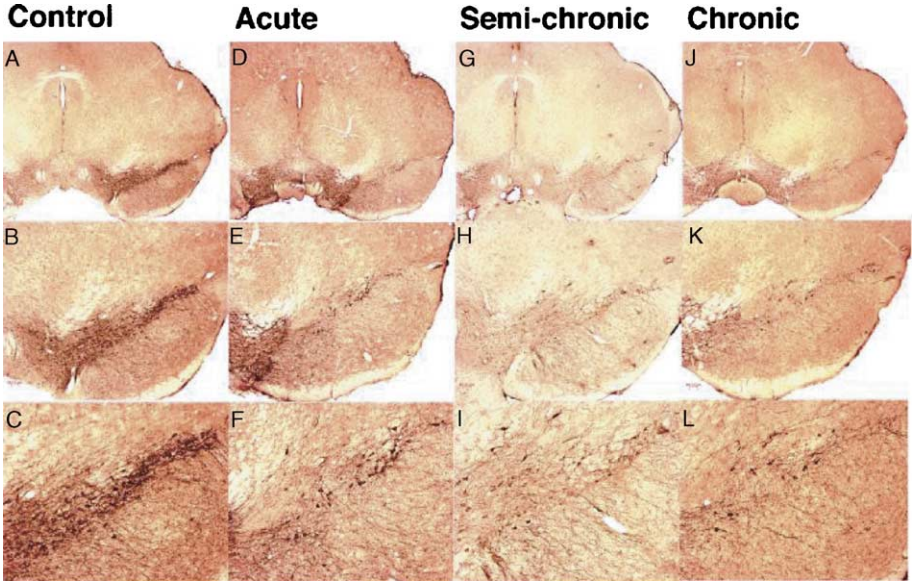

Fig. 2. Immunohistochemistry of mice brains for TH of the substantia nigra pars compacta following acute, semi-chronic and chronic MPTP treatments. (A–C) saline control, (D–F) acute, (G–I) semi-chronic and (J–L) chronic MPTP treatment. (A, D, G, and H) 2.5× magnification, (B, E, H, and K) 5× magnification, (C, F, I, and L) 10× magnification.

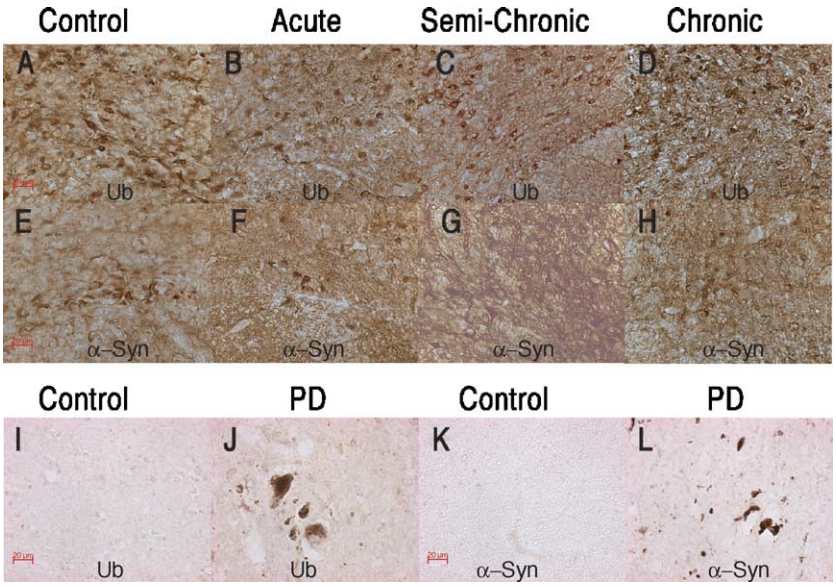

Fig. 3. Immunohistochemistry for (A–D, I and J) ubiquitin (Ub) and (E–H, K and L) α-synuclein (α-syn) in the substantia nigra pars compacta of mice (A–H) and human (I–L) brains at 40× magnification. (A and E) saline control, (B and F) acute, (C and G) semi-chronic and (D and H) chronic MPTP treated mice. (I and K) human non-PD control substantia nigra and (J and L) substantia nigra from a patient with PD.

α-synuclein aggregation and inclusion formation in cellular models [34]. Since MPTP leads to upregulation of α-synuclein mRNA and protein expression in DA neurons of the SN [40] and creates an environment of significant oxidative and nitrosative stress, we anticipated that MPTP would create an environment conducive to α-synuclein aggregation and inclusion formation. However, we failed to observe any α-synuclein accumulation or increased ubiquitin immunoreactivity following the three different MPTP regiments. Since another complex I inhibitor, rotenone, induces α-synuclein inclusions in rats following chronic administration, it may be that the acute and semi-chronic paradigms may kill DA neurons before α-synuclein has an opportunity to aggregate. However, even in the chronic 5-week MPTP injection paradigm and up to 6 months post-MPTP treatment, we failed to observed any α-synuclein inclusion formation, despite a prior report that suggested that this paradigm causes α-synuclein aggregation [32].

The MPTP mouse model has led to tremendous insight into the molecular mechanisms by which DA neurons in the substantia nigra die. The absence of detectable α-synuclein aggregates and formation of inclusions following MPTP intoxication is surprising, since α-synuclein aggregation is promoted, in part, through oxidative and nitrosative stress, and α-synuclein knockout mice are resistant to MPTP neurotoxicity [10]. The mechanism by which α-synuclein participates in MPTP toxicity is not known, but our data suggest that aggregation and formation of inclusions of α-synuclein following MPTP intoxication plays little if any role in MPTP toxicity. Since the absence of α-synuclein protects against MPTP intoxication, future studies will need to focus on mechanisms that do not involve α-synuclein aggregation and formation of inclusions.

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