Journal of the Neurological Sciences 392 (2018) 77–82

Contents lists available at ScienceDirect

Journal of the Neurological Sciences

journal homepage: www.elsevier.com/locate/jns

1-Methyl-4-phenyl-1,2,3,6 tetrahydropyridine/probenecid impairs intestinal motility and olfaction in the early stages of Parkinson's disease in mice

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ARTICLE INFO

Keywords:
Parkinson's disease
Early stage
Intestinal motility
Olfaction
1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine

ABSTRACT

Parkinson's disease (PD) is a progressive neurodegenerative disorder accompanied by movement deficits with selective degeneration of dopaminergic neurons in the substantia nigra (SN). Recent studies indicate that early diagnosis of PD has important implications for the disease-modifying strategy for PD showing not only some dopaminergic neuronal damage but also non-motor symptoms, which occur several years before the onset of motor symptoms. However, studies on the relationship between non-motor symptoms and its underlying mechanisms from the early to the late phase of PD are unknown. Here, we aimed to show alterations in the non-motor symptoms of PD, including colonic dysmotility and impaired olfaction, and the related factors by intraperitoneal injections of 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP) plus probenecid (MPTP/p). A mouse model of the early stage of PD was developed by systemic administration of MPTP (25 mg/kg, *i.p.*) and probenecid (100 mg/kg, *i.p.*) at 3.5-day intervals for a total of 10 injections. We performed motor and non-motor behavioral tests after 3 (called asymptomatic) and 10 (called symptomatic) injections of MPTP/p compared with the untreated (called control) group. We found that there were motor disturbances at the symptomatic stage, while impairments in intestinal motility and olfaction were observed from the asymptomatic stage. We also found the reduction of dopaminergic neuronal cell numbers in the SN and striatal dopamine transporter levels starting from the asymptomatic stage. At both asymptomatic and symptomatic stages, we demonstrated alterations in the expression of several proteins that are associated with non-motor deficits in the mouse ileum or olfactory bulb compared with the control group. Our findings in chronic MPTP/p-induced mice suggest their potential use as an animal model for the early stage of PD as well as a significant correlation between changes in relevant factors and symptoms.

1. Introduction

Parkinson's disease (PD), the second most common neurodegenerative disease, is characterized by motor deficits such as resting tremor, muscle rigidity, bradykinesia, and gait disturbance [1, 2]. These PD motor symptoms are caused by selective dopaminergic neuronal damage in the substantia nigra (SN) of the brain [3, 4]. Although the diagnosis of PD is entirely dependent on clinical motor symptoms caused by a dopamine deficiency, various prodromal non-motor symptoms such as several dysfunctions of the gastrointestinal (GI), olfactory, autonomic, and psychiatric systems arise at least 10 years before the onset of motor impairment [5–7]. It has been reported that autonomic dysregulations like drooling and increased heart rates as well as neuropsychological symptoms like sleep disturbances, mood disorders, and cognitive impairment are common features in early PD [8–10]. Among these non-motor deficits, impairments of the GI and olfactory functions are the most prevalent and predictable premotor symptoms experienced in approximately 60–70% of PD patients prior to the onset of motor signs [11]. Given this fact, precise diagnosis of PD non-motor symptoms is crucial for the development of disease-

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https://doi.org/10.1016/j.jns.2018.07.011
Received 10 January 2018; Received in revised form 28 June 2018; Accepted 12 July 2018

modifying therapies for PD. Therefore, there is great need for an animal model of PD that presents early non-motor symptoms as well as clinical motor symptoms.

Accumulated studies on preclinical models that can reproduce the symptoms and pathology of early PD have been reported by either genetic mutation or treatment with chemical neurotoxins such as 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). For example, mice with mutations in the leucine-rich repeat kinase 2 gene, which is one of the mutation forms in familial PD, exhibited olfactory loss and anxiety symptoms, but did not have dopaminergic neuronal damage in the SN [12]. Mice overexpressing human wild type α-synuclein under the Thy1 promoter displayed colonic motor dysfunction with no significant alterations in the related proteins, including choline acetyltransferase (ChAT), tyrosine hydroxylase (TH), neuronal nitric oxide synthase, and vasoactive intestinal peptide, in the colon [13].

In addition, it has been reported that alterations in the mRNA levels of phosphatidylcholine lipids or brain-derived neurotrophic factors in the SN were regarded as a pre-symptomatic marker following intracerebral injection of 6-OHDA into the SN of mice [14, 15]. Mice receiving intraperitoneal injections of MPTP also showed potential as an experimental model of the early stage of PD because of changes in the transcriptome profile of SNCA and the dopamine receptor 2 gene or activity of monoamine oxidase B in the SN and striatum (ST) [16, 17]. In particular, chronic administration of a low dose of MPTP with probenecid (MPTP/p) induced motor deficits accompanied by dopaminergic neuronal damage, gliosis, and α-synuclein inclusion in the SN [18]. However, these studies could not reproduce the non-motor symptoms and mainly focused on alterations in the brain due to genetic or neurotoxic insults.

In this study, we hypothesized that the most frequent non-motor events of PD, such as dysfunctions of the GI and olfactory systems, could be reproduced by MPTP/p administration. Moreover, we explored the underlying mechanisms, including changes in the regulatory proteins that are related to intestinal motility and olfaction from the early to the late phase of PD induced by MPTP/p.

2. Materials and methods

2.1. MPTP/probenecid-induced PD model

Male 10-week-old C57BL/6 mice were purchased from Central Lab Animal Inc. (Seoul, South Korea). The animals were housed (n = 4 per cage, 2 cages per group) at an ambient temperature of 23 ± 1 °C and relative humidity of 60 ± 10% under a 12-h light/dark cycle and were allowed free access to water and food. This model was established according to our previously reported methods [19]. Mice were divided into 3 groups according to the number of MPTP/p administrations: Group 1, control (without MPTP/p injection); Group 2, asymptomatic (3 injection times of MPTP/p); Group 3, symptomatic (10 injection times of MPTP/p). Briefly, all mice except the control group were injected with MPTP hydrochloride (25 mg/kg/day in saline, *i.p.*) along with probenecid (100 mg/kg/day in 5% $NaHCO_3$, *i.p.*). Probenecid was administered 30 min prior to MPTP injection as it induces a chronic PD model via reducing the clearance of MPTP and increasing the rate of passage to the blood-brain barrier [20–22]. Mice received a total of 10 injections of MPTP in combination with probenecid at an interval of 3.5 days.

2.2. Behavior test

2.2.1. Open field test

The open field test is a useful method to measure ambulation ability in mice [23]. We performed the test between 9 p.m. and 2 a.m. to avoid diurnal variation. Mice were placed in the testing chamber (40 × 25 × 18 cm) with white floors, followed by a 30-min recording period using a computerized automatic analysis system (Biobserve, Germany). The data collected by computer included the total distance traveled by tracking the center of the animal.

2.2.2. Rotarod test

The rotarod test is a useful method for measuring motor coordination in a mouse model of PD [24]. The rotarod unit consists of a rotating spindle (7.3 cm diameter) and five individual compartments. After two times of training (8–10 rpm rotation speed), the rotation speed was increased to 12 rpm in a test session. The time each mouse remained on the rotating bar was recorded over two trials per mouse with a maximum length of 3 min per trial. Data are presented as the mean time on the rotating bar over the two trials.

2.2.3. Bead expulsion test

The bead expulsion test was performed for monitoring intestinal motility [25, 26]. A plastic bead (diameter, 3 mm) was inserted into the colon at a distance of 2 cm from the anal verge. The time required for expulsion of the bead was measured and taken as an estimate of intestinal motility.

2.2.4. Buried pellet test

The buried pellet test is a useful method to examine olfactory deficits in the early stages of PD [27, 28]. Mice were food-deprived for 20 h before the test. The test was conducted in a clean plastic cage (24 × 42 × 15 cm). A cheese-smelly pellet was buried 1 cm under the bedding in a cage corner, and the mouse was positioned in the center of the cage. The time spent to bite the pellet was measured at a maximum trial length of 5 min per mouse.

2.3. Tissue preparation

The mice were sacrificed after all behavior tests were finished at 3 and 10 injections of MPTP/p, respectively. For immunohistochemical studies, the mice were anesthetized and transcardially perfused with 0.05 M PBS, and then fixed with cold 4% paraformaldehyde (PFA) in a 0.1 M phosphate buffer. The brains were quickly removed and post-fixed in a 0.1 M phosphate buffer containing 4% PFA overnight 4 °C and then immersed in a solution containing 30% sucrose in 0.05 M PBS for cryoprotection. Serial coronal sections that were 30 μm-thick were cut on a freezing microtome (Leica, Germany) and stored in cryoprotectant (25% ethylene glycol, 25% glycerol, 0.05 M phosphate buffer) at 4 °C. For western blotting analysis, the mice were decapitated and the ileum tissues were isolated and stored at −80 °C until use.

2.4. Western blotting

Ileum tissues were lysed with a triple-detergent lysis buffer. The lysates were separated by 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis, and gels were processed for antigens and blotted onto immobilon-P transfer membranes for 1 h 30 min. Membranes were incubated with 5% skim milk in a mixture of tris-buffered saline and Tween 20 for 1 h and then with the primary antibodies (TH 1:1500, inducible nitric oxide synthase (iNOS) 1:1500, α-synuclein 1:2000, and β-actin 1:3000) overnight at 4 °C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h. Blots were detected using an enzyme-linked chemiluminescence detection kit, and an LAS-4000 mini system (Fujifilm Corp., Japan) was used for visualization. The intensities of the bands were normalized to the β-actin band using Multi Gauge software (Fujifilm Corp., Japan).

2.5. Immunohistochemistry

Brain sections were taken from the each brain region: between bregma −3.16 mm and bregma −3.64 mm (SN), between bregma