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Role of Rho-mediated ROCK-Semaphorin3A signaling pathway in the pathogenesis of Parkinson's disease in a mouse model

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ABSTRACT

Objective: The present study aims to elucidate the role of Rho-mediated ROCK-Semaphorin3A signaling pathway in the pathogenesis of Parkinson's disease (PD) in a mouse model.
Methods: One-hundred twelve eight-week male C57BL/6 mice were selected. The mouse model of PD was constructed by intraperitoneal injection of MPTP. All mice were divided into four groups (28 mice in each group): Blank group, Model group, Rho knockout (Rho+/−) group and ROCK knockout (ROCK+/−) group. Changes of behavior of the mice were studied through automatic moving test and rotarod test. Immunohistochemistry (IHC) was used to detect the expressions of TH, CD11b and GFAP. High performance liquid chromatograph (HPLC) was performed for detection of dopamine and its metabolic product. The mRNA and protein expressions of Rho, ROCK, Sema3A, PlexinA and NRP-1 were detected using quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting.
Results: Rho and ROCK knockout improved the damage caused by MPTP on the behavior of mice and protected dopaminergic neurons from injury, along with the increases of dopamine and its metabolic product. The mRNA and protein expressions of Rho, ROCK, Sema3A, PlexinA and NRP-1 were increased in PD mice in the Model group compared with those in the Blank group. Compared to the Model group, the mRNA and protein expressions of Rho, ROCK, Sema3A, PlexinA and NRP-1 were reduced in the Rho+/− and ROCK+/− groups.
Conclusion: These findings indicate that Rho and ROCK knockout may improve the behavior of mice and prevent MPTP-induced dopaminergic neurons damage by regulating Sema3A, PlexinA and NRP-1 in a mouse model of PD.

1. Introduction

Parkinson's disease (PD) is an age-related manifestation of central nervous system lupus that ranks only second behind Alzheimer's disease in prevalence, resulting in social and economic burden [1]. PD has been considered as an incurable and progressive disorder which gradually robs the individual of motor control [2]. PD is pathologically characterized by body tremors, slowed movement, rigidity and postural instability, asymmetric bradykinesia and even non-motor extra-nigral symptoms [3,4]. It has been reported that approximately 1% of individuals aged >65 years suffer from AD across the world [3]. Traditionally, the confirmation of PD is still greatly according to the detections of its clinical and autopsy features [5]. Unfortunately, there is no sufficient diagnostic biomarker of PD even though current interventions (genetic, olfactory, and neuroimaging studies) have been recommended for diagnosis [6]. Antiparkinsonian drugs may potentially effectively control motor symptoms in the early stages of the disease but fail to halt progression, and only insufficiently address functional impairments at the later stages [7]. Recently, there is accumulating evidence that Rho-associated protein kinase/Rho-kinase (ROCK) signaling pathway might be responsible for the cellular survival and management of neurological disorders [8,9].

ROCK belongs to a downstream effector protein of the Rho GTPase, which are the number of a globular, monomeric group of small signaling G-protein molecules [10]. ROCK signaling pathway participates in the pivotal neuronal processes in axonogenesis, stability of synapses, as well as growth cone dynamics [8]. Surprisingly, Rodriguez-Perez et al. revealed that Rho kinase inhibitors is considered as a neuroprotective strategy, which may be an effective adjuvant to cell therapy in PD [9,11]. Lars et al. also confirmed that ROCK functions as a potential pharmacologic target for the management of neurotraumatic and neurodegenerative diseases [12]. Semaphorin3A (Sema3A) belongs to class 3 semaphorins which are phylogenetically conserved guidance proteins that are responsible for angiogenesis, branching morphogenesis, axon growth and cell migration [13,14]. Sema3A is known to result in neuronal apoptosis and functions as a chemorepellent factor for axonal growth. Furthermore, Yasuhara et al. suggested that Sema3A is toxic

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to cultured dopaminergic neurons at very high dosages [15]. It has been found that Sema3A signals are regulated by a receptor complex consisting of neuropilin1 (NRP-1) and PlexinA, Sema3A binding and signaling receptors, respectively [16]. Currently, little data with regard to Rho/ROCK-Sema3A association has been available. Here we hypothesize that Rho and ROCK knockout can alleviate the damage on behavior and protect dopaminergic neurons from injury by modulating Sema3A and its two receptors of PlexinA and NRP-1 in a mouse model of PD.

2. Materials and methods

2.1. Experimental animal and grouping

A total of 112 eight-week male C57BL/6 mice of SPF level were purchased from Shanghai Laboratory Animal Center of Chinese Academy of Sciences (Shanghai, China). These mice were divided into four groups (28 mice in each group): Blank group, Model group, Rho knockout (Rho +/−) group and ROCK knockout (ROCK +/−) group. All mice were given seven days for adaption, treated with sufficient food and water, with 4 mice in each cage. All experimental procedures were conducted in accordance with animal care principles and guidelines of National Institutes of Health (NIH).

2.2. Construction of a mouse model of PD

The mice in the Model, $Rho^{+/-}$, and $ROCK^{+/-}$ groups were injected with 30 mg/kg 1, 2, 3, 6-tetrahydro-1-methyl-4-phenyl-pyridin (MPTP) (Sigma-Aldrich Co. Ltd., St. Louis, Missouri, the United States) at 9 A.M. for 28 days consecutively until behavior test was terminated. Following behavior test, the mice were killed by cervical dislocation and brain tissues were used for follow-up experiment. The mice in the Blank group were subjected to intraperitoneal injection of normal saline with the same volume.

2.3. Behavior evaluation of PD mice

Rotarod test was performed before MPTP injection and 7, 14, 21, and 28 days after MPTP injection. At 3 days before MPTP injection, a period of time was provided for mice to get used to the rotarod system (Columbus Instruments, Columbus, Ohio, USA) and the data were excluded from statistical analysis. After 30 s adaption, the rotarod system started at 5 rpm/min for 30 s, after which the speed was increased to 0.1 rpm/s. When the mice fall off, the timer was stopped. The time when mice stayed on the rotarod system after speeding up was recorded as the results of the test. Each mouse was tested for three times with an interval of 15 min.

Automatic moving test was performed before MPTP injection and 7, 14, 21, and 28 days after MPTP injection using an automatic moving tester (Beta Lasermike Inc., Dayton, OH., USA) in quiet and dark environment. The test was conducted during 9–11 A.M. The test box was 40 cm × 40 cm and three infrared emission devices were put in different directions, sensing the automatic activities route of mice. Half an hour was provided for adaption, after which test started and lasted for 30 min. The whole process was monitored by computer and infrared sensor was applied to output the time of moving, rest and distance. The moving time and total distance were obtained for statistical analysis.

2.4. Preparation of brain tissue specimens

The ice box, dry ice box, six-well plate, 2 ml centrifuge tube, freezing tubes, phosphate buffered saline (PBS, containing NaCl: 8 g, Na_2HPO_4: 1.1 g, KCl: 200 mg; KH_2PO_4: 200 mg, pH = 7.3) and head/brain model box of mice were prepared for specimens collection. At 28 days after MPTP injection, the head of mice was cut down and soaked in PBS. The brain tissue was taken and midbrain was cut down along the coronal plane from the head in model box. Then, the left and right striatum were separated, put into 2 ml centrifuge tube, stored in liquid nitrogen immediately overnight and transferred into −80 °C refrigerator for storage on the second day. The midbrain was sliced into two parts. The right side was put in 2 ml centrifuge tube, stored in liquid nitrogen immediately overnight and transferred into −80 °C refrigerator for storage on the second day. The left side was fixed with 4% paraformaldehyde for 24 h after which the paraformaldehyde was poured out, and 15% and 30% sucrose were added for 12 h dehydration respectively. The paraffin was used to embed the dehydrated midbrain and fixed on the microtome (MICON, Germany). Coronally sliced with 30 μm interval, the samples were put into a 6-well plate containing PBS, numbered and stored in 4 °C refrigerator. The brain slices of mice in each group were randomly selected for fluorescence quantitative polymerase chain reaction (PCR) (n = 10), for Western blotting (n = 10), and for immunohistochemistry (IHC) (n = 10).

2.5. Immunohistochemistry (IHC)

Brain tissue slices were obtained, baked at 65 °C for 2 h, dewaxed in xylene for 10 min, subjected to gradient ethanol elution and washed up by distilled water for 3 times (2 min/time). The slices were repaired by high pressure in Tris-EDTA buffer solution (PH 9.0, Boster BioTec, Wuhan, China), washed by distilled water, totally soaked in 3% H_2O_2 and incubated in the dark at room temperature for 10 min. PBS (Boster BioTec, Wuhan, China) was used to wash the slices. Normal goat serum was added onto the slices at 37 °C and incubation of slices was performed for 10 min. At 1:100, tyrosine hydroxylase (TH), integrin alpha M (CD11b), glial fibrillary acidic protein (GFAP) primary antibodies (Abcam, Cambridge, England), attenuated by polybutylene (PB), were added. The samples were incubated at 4 °C refrigerator for 24 h, taken out on the next day and washed by distilled water. Secondary antibody of rabbit anti-mouse solution (Boster BioTec, Wuhan, China) was added into the slices, which were incubated at 37 °C for 30 min and washed up by distilled water. Dyed by diaminobenzidine (DAB), the samples were re-dyed by the hematoxylin for 1–2 min (Boster BioTec, Wuhan, China). Hydrochloric acid alcohol differentiation, back to blue in water, the differentiation of dyeing was observed under microscope. After dehydration of gradient ethanol, the neutral balsam was sealed. Microscope (OLYMPUS Corporation, Japan) was used to take photos. Interpretation of results: tan or sepia particle in the IHC of TH, CD11b, glial fibrillary acidic protein (GFAP) implied positive. Five visions were randomly selected under high power lens (400×) and positive cells of each group were calculated.

2.6. High performance liquid chromatograph (HPLC)

Before HPLC (Eicom, Japan) detection, moving phase was used to maintain the balance of chromatographic column for 2 h or overnight. Corpus striatum stored at −80 °C was weighed, according to which corresponding dosage of perchloric acid (0.1 mol) was added. The samples underwent low temperature ultrasound pyrolysis. After 30 min, the samples were centrifuged for 15 min at 14,000 × *g* in refrigerated centrifuge, and the supernatant was obtained. The supernatant, filtered by 0.25 μm filter, was injected into stable Bond of HPLC. The expression of dopamine (DA), 5-the tryptamine (5-HT) and its metabolite, dihydroxy phenylacetic acid (DOPAC), herpes virus ateles (HVA) and hydroxy indole acetic acid (5-HIAA) were detected by high performance liquid chromatography (HPLC) method.

2.7. Quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA was extracted from the right side of midbrain stored at −80 °C in accordance with RNA extraction kit (Promega Biotech Co., Ltd., Madison, Wisconsin, USA) specification. The ultraviolet spectrophotometer was used to testify the optical density (OD) 260/280 ratio