

c-Jun N-terminal kinase 3 signalling serves a potential role as a biomarker for determining the pathogenesis of Parkinson's disease

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Abstract. Parkinson's disease (PD) is a movement-associated disorder that specifically affects dopamine-producing neurons. The disease causes demyelination that adversely impacts upon the motor activity of the brain. Currently there are no promising biomarkers for PD; improved understanding of the molecular mechanisms underlying the different pathological stages of PD are required to enable identification of a novel biomarker. The present study successfully established a PD mouse model via nasal injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. The expression of c-Jun N-terminal kinase 3 (JNK3) and caspase-3 in two different pathological stages of PD were analysed using immunohistochemistry and western blot analysis. The results indicated that the initial PD pathogenesis recovers on response to rasagiline. Immunohistochemistry and western blot analysis revealed that treatment with rasagiline positively regulated early-stage PD pathogenesis by downregulating the expression of JNK3 and upregulating caspase-3; however, there was no positive effect on the advanced stages of PD. Overall, these results concluded that rasagiline has the ability to inhibit the expression of JNK3 and upregulate caspase-3 in early stages of PD; however, rasagiline appears to have no impact on JNK3 and caspase-3 levels in the advanced stages of PD.

Introduction

Parkinson's disease (PD) is a slow-progressing neurological disease that results in degeneration of nerve cells (1). As a result, the majority of patients exhibit signs of dementia in the earlier stages (2), and when the disease progresses they start to exhibit clinical symptoms including tremor, bradykinesia, positional imbalance and rigidity (3). Ageing is a key factor in the development of PD, as it results in morphological and neurochemical

alterations in dopamine-producing neurons (4-6). Treating PD in earlier stages has a neuroprotective role in preventing or prolonging the development of disease complications (7). Treatment at later stages of PD currently has no effect, and in most cases it results in treatment resistance (1).

Earlier detection of PD is essential in the clinical management of PD. Physiological symptoms occur following neural degeneration, and thus, suitable biomarkers that detect earlier stages of PD need to be identified (8). Currently available biomarkers fail to specifically identify PD; further complications include variation within individual results and a lack of experimental reproducibility (8-10). To identify an effective biomarker it is helpful to understand the pathogenesis of PD development; however, the underlying mechanism underlying the neural degeneration of PD and its associated neural dysfunction remain to be clearly elucidated (1). An improvement in the basic knowledge of this disease is required to improve understanding of its pathogenesis, and to aid in the identification and validation of biomarkers associated with different pathological stages.

C-Jun N-terminal kinase 3 (JNK3) is markedly expressed in brain tissue (11) and has demonstrated roles in inflammation, apoptosis, cellular proliferation and neural degeneration (12,13). The present study used PD mouse models to investigate the role of apoptosis and JNK3 in PD, and how they associate with different two pathological stages of this disease.

Materials and methods

Mouse model with PD. Initially, 30 BALB/c male mice (age, 6 weeks; weight, 30-40 mg) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) were used in the present study. Mice were treated twice daily with MPTP and maintained under a 12 h light/dark cycle at 25±1°C with 45±5% relative humidity and food and water provided freely. The PD mouse model was developed using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which was directly administered into the nasal cavity of the mice at a dosage of 1.5 mg/nostril (total dose, 60 mg/kg) (14). Following treatment with MPTP, the mice displayed early symptoms of PD by the 2nd week, including hind limb weakness and restricted movements. On the 4th week, mice exhibited advanced symptoms of PD with two hind limb paralyses. Limb weakness was used to assess the development of Parkinson's disease (15). The animals,

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chemical used and the experimental methods for developing the PD model were approved by the animal care board of The First People's Hospital of Shangqiu (Shangqiu, China).

Treatment with rasagiline. The mice were divided into the following experimental groups ($n=6/\text{group}$): Normal (untreated); PD early; PD advanced; PD early + rasagiline; PD advanced + rasagiline. Mice with early and advanced PD were treated with rasagiline by dissolving the drug in water and administering orally at a dose of 1 mg/day (30 mg/kg), for one week. Water was used as a vehicle control. Following treatment, the clinical symptoms including limb movement and brain histological variation of the mice were analysed. The mice were subsequently sacrificed and whole brain samples were removed for further investigation.

Histology and immunohistochemistry. Brain samples obtained from early and advanced stages of PD and samples from untreated mice were sectioned into smaller pieces, fixed with 10% formaldehyde and embedded in paraffin. Formalin-fixed paraffin embedded tissue samples were subjected to histological and immunohistochemical analysis (16). Using a microtome, tissue samples were evenly sliced into 5 μm sections and placed on a slide warming table. Following deparaffinization and rehydration, the slides were stained with haematoxylin and eosin for histopathological analysis. For immunohistochemistry, the endogenous peroxidase activity was blocked using 10% H_2O_2 and nonspecific binding was blocked in 4% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) solution for 1 h. The tissue sections were incubated with an anti-JNK3 antibody (cat. no. ab126591; Abcam, Cambridge, UK; 1:500) or with an anti-caspase-3 antibody that specifically detects cells with early apoptosis (cat. no. ab13847, Abcam; 1:500) overnight at 4°C. Following washing with PBS twice to reduce non-specific binding of the primary antibody, the tissues sections were incubated with a polyclonal goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (ab6721, Abcam; 1:5,000) for 1 h at room temperature. The slides were then washed and developed using diaminobenzidine solution and counterstained with haematoxylin and eosin. The experiments were repeated three times and the signals obtained were examined and documented under a light microscope equipped with NIS-Elements Viewer 4.3 (Nikon, Corporation, Tokyo, Japan).

Western blot analysis. To analyse the differential expression of JNK3 in samples obtained from control, and PD treated and untreated mice, western blot analysis was performed. The samples were dissected out and crushed on ice along with 2X sample buffer [500 mM Tris (pH 6.8), 20% glycerol, 4% SDS, 5% β -mercaptoethanol, 0.1% bromophenol blue], then the tube are heated in a boiling water bath for 5 min to obtain the protein lysate. The extracted protein samples were measured using the Lowry method and 70 $\mu\text{g}/\text{well}$ was separated by 10% SDS-PAGE according to a previously described protocol (17). Separated proteins were transferred to polyvinylidene difluoride membranes, and the membranes were blocked with 4% BSA solution to limit non-specific binding of the antibody. Membranes were subsequently incubated with an

anti-JNK3 primary antibody (cat. no. ab126591, Abcam; 1:500) or an anti-caspase-3 antibody (cat. no. ab13847, Abcam; 1:500) overnight at 4°C. Actin (cat. no. ab3280, Abcam; 1:500) served as a loading control. Following incubation, the membrane was washed three times with TBS-Tween 20 (TBST) buffer and incubated with a goat anti-rabbit IgG alkaline phosphatase-conjugated secondary antibody (Abcam, ab97048; 1:5,000) for 1 h at room temperature. The membrane was subsequently washed in TBST and developed with 5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma-Aldrich; Merck KGaA) to obtain the signal.

Results

Histological differences between healthy, PD and rasagiline-treated brain tissue. Histological examination of brain tissue was performed to assess the pathological stages of PD mice. The analysis of histopathological features is useful in understanding the etiology of disease progression, which other techniques, such as magnetic resonance imaging, cannot provide. Analysis of the control brain tissue revealed cell bodies and axons that were scattered uniformly throughout the tissue (Fig. 1A). The mice treated with MPTP for 2 weeks developed early stages of PD, with the development of lesions throughout the brain tissue (Fig. 1B); the affected mice demonstrated hind limb weakness. Similarly, the mice treated with MPTP for 4 weeks exhibited poor movement, with paralyse of both hind limbs, representing an advanced stage of PD. Histological analysis of brain tissue from the advanced-stage PD mice revealed extensive lesions that severely affected the white matter, which is important for motor activity (Fig. 1C).

Following treatment with rasagiline for one week, the early-stage PD mice exhibited improvement in their hind limb weakness. These mice demonstrated active movement and histological examination revealed that the brain lesions were reduced in number, compared with the untreated early-stage PD mice (Fig. 1D). Mice with advanced-stage PD presented no clinical or histological improvements. Furthermore, the two hind-limb paralyse deteriorated clinically with progressive restricted in limb movement. The brain tissue also exhibited more degenerative tissue, potentially preventing the mice from recovering (Fig. 1E).

JNK3 expression at different pathological stages of PD. Inflammatory responses and various stress stimuli can activate JNK3, which in turn has the ability to regulate important cellular activities including cell differentiation, survival and death (18). The present study analyzed JNK3 expression at two different pathological stages of PD using immunohistochemistry. Analysis of brain tissue from control mice revealed very mild expression of JNK3 (Fig. 2A), due to the regulation of JNK3 expression. Following treatment with MPTP for 2 weeks, the JNK3 signal demonstrated upregulation (Fig. 2B), which may have been due to MPTP-induced neurological stress. Following treatment with MPTP for 4 weeks, the advanced-stage PD brain tissue revealed elevated JNK3 expression when compared with the early PD brain tissue (Fig. 2C).

JNK3 expression was investigated following treatment with rasagiline, which has the ability to reversibly

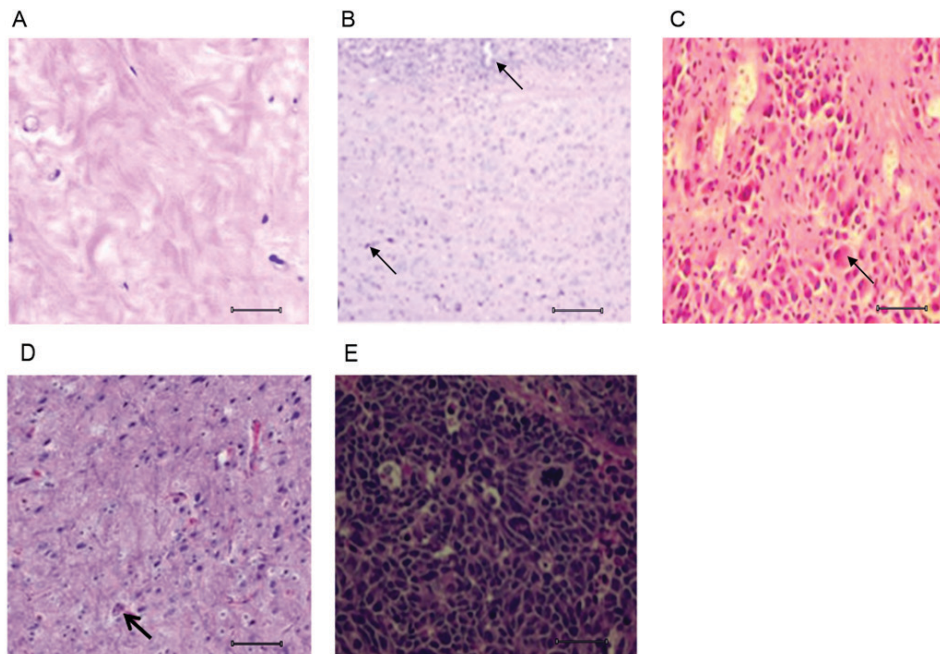


Figure 1. Histological differences between healthy, PD and rasagiline-treated brain tissue. (A) Untreated control brain tissue with axons, cell bodies and with myelination that are uniformly distributed. Histological analysis of (B) early-stage PD tissue with lesions, (C) advanced-stage PD tissue with extensive lesions, (D) early-stage PD mice treated with rasagiline, exhibiting recovery to damage, and (E) advanced-stage PD mice treated with rasagiline, revealing no effect of rasagiline. Arrows indicate lesions. Scale bar=100 μ m. PD, Parkinson's disease.

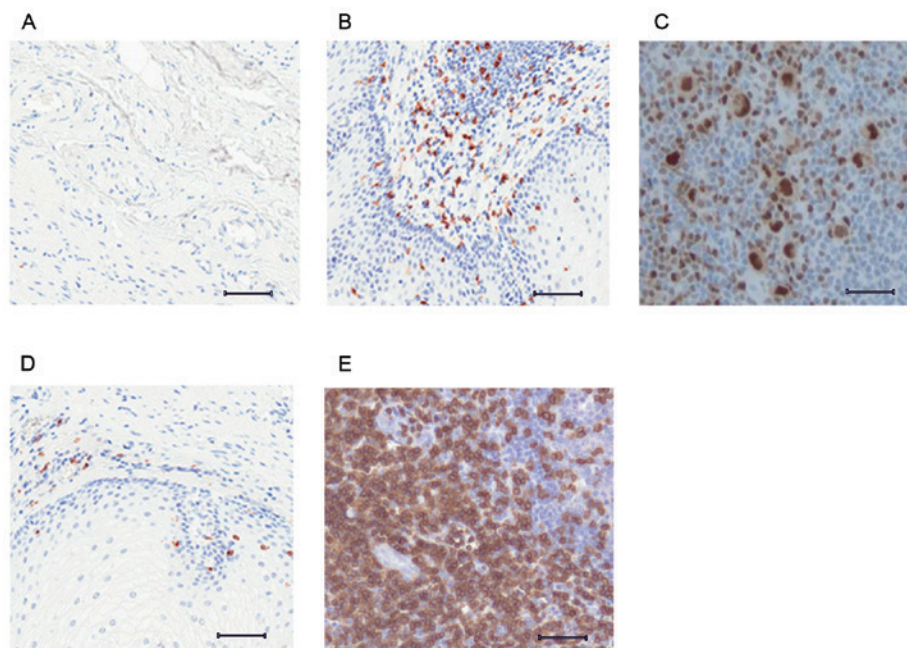


Figure 2. Immunohistological differences associated with JNK3 expression and response to rasagiline. Immunohistological analysis of (A) control brain tissue, revealing minimal expression of JNK3, (B) early-stage PD brain tissue, with marginal expression of JNK3, (C) advanced-stage PD brain tissue, with elevated expression of JNK3, (D) early-stage PD mice treated with rasagiline, revealing downregulated expression of JNK3, and (E) advanced-stage PD mice treated with rasagiline, revealing high expression of JNK3. Scale bar=100 μ m. JNK3, c-Jun N-terminal kinase; PD, Parkinson's disease.

inhibit the enzyme monoamine oxidase-B (19). Rasagiline exhibited marked inhibitory effects on JNK3 expression in early-stage PD (Fig. 2D). However, rasagiline treatment of mice with advanced-stage PD had no effect, with the brain tissue exhibiting higher JNK3 expression compared with the untreated advanced-stage mice (Fig. 2E).

Association between caspase-3 and JNK3 expression. In multicellular animals, apoptotic signals serve to regulate the elimination of abnormal cells (20). Caspase-3 expression was assessed in the brain tissue of early- and advanced-stage PD mice, to investigate a potential correlation in expression levels between caspase-3 and JNK3, in early and advanced PD pathological stages. The control mice revealed minimal

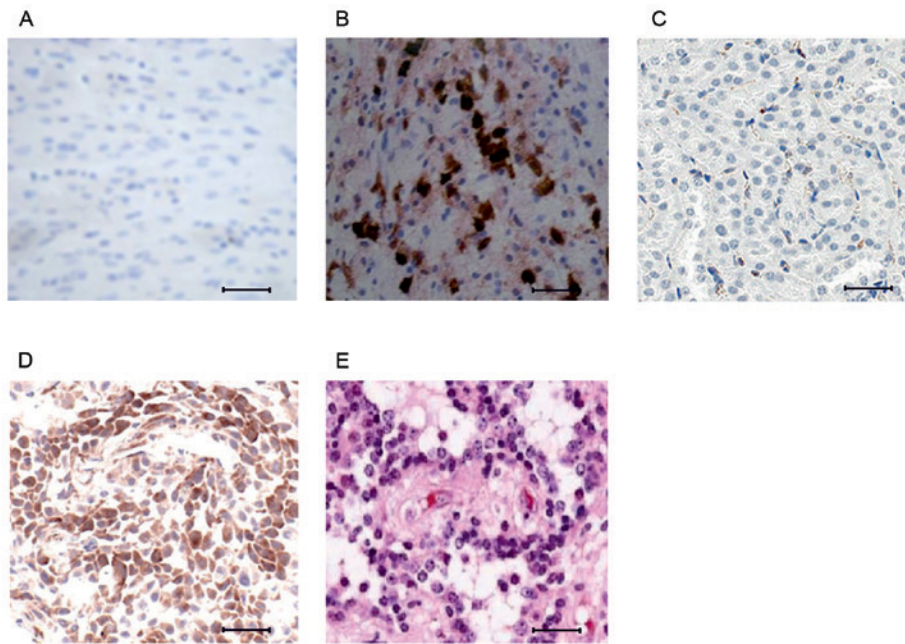


Figure 3. Immunohistological analysis of caspase 3 expression and response to rasagiline. Immunohistological analysis of (A) control brain tissue, revealing minimal expression of caspase-3, (B) initial PD brain tissue, demonstrating elevated expression of caspase-3, (C) advanced stage PD brain tissue, revealing downregulated expression of caspase-3, (D) initial PD mice treated with Rasagiline demonstrating upregulated expression of caspase-3 when compared with untreated initial PD brain tissue, (E) advanced stage PD mice treated with Rasagiline indicating downregulated expression of caspase-3. Scale Bar=100 μ m size. PD, Parkinson's disease.

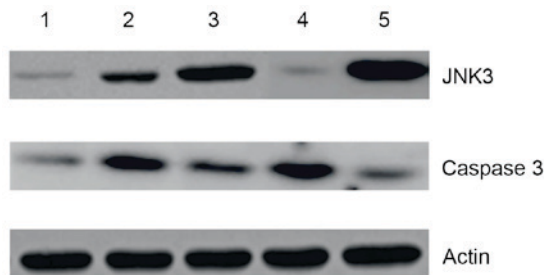


Figure 4. Western blot analysis of JNK3 and caspase-3 expression. Expression of (A) JNK3 and (B) caspase-3, as determined by western blot analysis. Lanes 1-5 represent expression in control, early-stage PD, advanced-stage PD, early-stage PD mice + rasagiline and advanced-stage PD mice + rasagiline groups. Actin served as a loading control. JNK3, c-Jun N-terminal kinase; PD, Parkinson's disease.

expression of caspase-3 (Fig. 3A). Early-stage PD mice exhibited a marked increase in caspase-3 expression (Fig. 3B); however, caspase-3 expression was downregulated in the brain tissue of advanced-stage PD mice (Fig. 3C). Rasagiline-treated early-stage mice exhibited higher levels of caspase-3, compared with the untreated early-stage mice (Fig. 3D), however, the advanced-stage PD mice demonstrated minimal expression of caspase-3 (Fig. 3E).

Western blot analysis of JNK3 and caspase-3 expression. The immunohistochemistry results were further investigated using western blot. Expression of JNK3 and caspase-3 was analysed, as presented in Fig. 4A and B. The results supported the expression patterns observed by immunohistochemical analysis. JNK3 demonstrated elevated expression with progression of PD. Rasagiline treatment was only able to reduce the level

of JNK3 in early-stage PD (Fig. 4A). In contrast, caspase-3 exhibited elevated expression in early-stage PD (Fig. 4B), compared with advanced-stage PD tissues.

Discussion

Damage to the white matter of the brain is particularly associated with PD; in advanced stages this results in demyelination, which severely impacts upon the motor activity-signalling capacity of the neuron (21). The damage that occurs in earlier stages of PD may be postponed using novel drugs; however, advanced stages of PD are difficult to control (22). The present study investigated the expression pattern of JNK3 in two different pathological stages of PD and compared them with the expression of caspase-3. Compared with early-stage PD mice, greater clinical and histological deterioration was observed in advanced-stage PD mice (Fig. 1A-C). The early-stage PD mice treated with rasagiline demonstrated clinical and histological improvements; however, there was no effect on advanced-stage PD mice. This data indicated that rasagiline may not be able to reverse neural degeneration in advanced-stage PD.

Histopathological and immunohistochemical analysis of different PD pathology stages, using a mouse model, may provide enhanced understanding of PD pathology, due to the ability to investigate pathological stages that are difficult to analyse with other imaging techniques (23). In the present study, a mouse model of PD was used to determine the impact of rasagiline treatment in two different pathological stages of PD.

JNK regulates cell growth, cell survival and apoptosis, and has demonstrated a role in testicular (24) and breast (25) cancer; however, the role of JNK3 on apoptosis has received limited research attention. Apoptosis is a fundamental mechanism that

regulates the survival of abnormal or damaged cells (26). The results of the present study indicated that the expression of JNK3 may be associated with PD disease progression. Notably, following treatment with rasagiline, expression of JNK3 was only observed in early-stage PD; however, no effect on advanced-stage PD was observed. The expression of caspase-3 was elevated in early-stage PD compared with control and advanced-stage PD. Following rasagiline treatment, caspase-3 was highly upregulated in early-PD samples, compared with advanced stage PD. Similar results were obtained by western blot analysis.

In conclusion, as PD progresses, it results in the accumulation of lesions with axonal and cell body damage. The clinical manifestation and histological damage observed in early-stage PD may be improved by treatment with rasagiline. Furthermore, rasagiline may have the ability to inhibit the expression of JNK3 and upregulate the expression of caspase-3 in early-stage PD; however, it has no effect in advanced stages of PD. The results of the present study may help to further understand the complexities of this disease, and may aid the discovery of novel therapeutic drugs to treat PD.

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