Axonal Degeneration of Nigra-striatum Dopaminergic Neurons Induced by 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine in Mice

L-H Li[1,2], H-Z Qin[1], J-L Wang[1,2], J Wang[1,2], X-L Wang[1,2] and G-D Gao[1,2]

[1]Department of Neurosurgery, Tangdu Hospital, Fourth Military Medical University, Xi'an City, China; [2]Institute of Functional Brain Disorders, Fourth Military Medical University, Xi'an City, China

As one of the main pathological changes of Parkinson's disease (PD), axonal degeneration was thought to be a passive process that is secondary to the apoptosis of dopaminergic neurons and, therefore, it has been overlooked for some time. Recent research, however, has indicated that axonal injury is the first location of damage in dopaminergic neurons in PD, and that the degree of injury in axonal degeneration is higher than in neural death. This study explored the relationship between apoptosis of dopaminergic neurons and their axonal degeneration by observing dopaminergic neuronal injury and axonal degeneration in the substantia nigra-striatum in different animal PD model and control groups. The results show that axonal degeneration plays a crucial role in the pathogenesis of PD and suggest that the process of axonal degeneration occurs independently of apoptosis and may even induce neuronal apoptosis. Thus, preventing axonal degeneration may be a potential new therapeutic strategy for PD.

KEY WORDS: Parkinson's disease; Axonal degeneration; Apoptosis microtubule; Pathogenesis

Introduction

Parkinson's disease (PD) is one of the common degenerative diseases of the central nervous system. It is traditionally recognized that apoptosis of dopaminergic (DA) neurons is one of the main pathological manifestations of PD, while axonal degeneration is viewed as a passive process that is secondary to apoptosis. The importance of axonal degeneration has, therefore, often been overlooked. Recent research, however, has found that inhibition of neuronal apoptosis alone cannot effectively postpone the pathogenesis of PD,[1] suggesting that there are other causes of neuronal death in addition to apoptosis that either occur earlier or are independent of apoptosis. Injury to axons and synapses in the striatum has been shown to precede the loss of DA neurons in the substantia nigra both in PD patients and in animal models of PD.[2] Axonal injury that is not rectified may cause axonal degeneration

and even 'dying-back' neuronal degeneration,[2,3] with 80 – 90% of tyrosine hydroxylase (TH)-positive terminals being lost in the striatum together with 50 – 60% of DA neurons being lost in the substantia nigra.[1] This suggests that dysfunction of the axons and synapses of the DA neurons occurs earlier than that of the DA neurons themselves. Thus, axonal degeneration may be one of the main causes of PD and preventing it could be a therapeutic target in treating PD.

This study explored the relationship between apoptosis of DA neurons and their axonal degeneration by observing the injury of DA neurons and axonal degeneration in the substantia nigra-striatum in different groups of mice.

Materials and methods
ANIMALS
Male C57 black (C57BL) mice weighing 25 – 27 g and aged 8 weeks were obtained from the Animal Centre of the Fourth Military Medical University, Xi'an City, China. The Ethics Committee of the Fourth Military Medical University approved the study protocol, and all efforts were made to minimize the suffering of animals and the number of animals used. During the experiment, the mice were housed in a temperature- and humidity-controlled vivarium with a 12-h light/dark cycle. All experiments were performed during the daytime. Food and water were available *ad libitum*. Animals were randomly divided into three groups: the control group (saline), an acute PD model group, and a sub-acute PD model group.

TREATMENTS
Mice in the acute PD model group received a total of four intraperitoneal (IP) injections each of 20 mg/kg 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) at 2-h intervals and were sacrificed on days 1, 3, 5 and 7

after the first injection. Animals in the sub-acute PD model group received IP injections of MPTP 36 mg/kg once daily for 7 days and were sacrificed 7 days after the first injection. Animals in the control group were given IP injections of saline (1 ml) at the same times as MPTP was given to the acute and sub-acute groups and were sacrificed on days 1, 3, 5 and 7.

TISSUE PREPARATION
After behavioural testing, which involved pole-jumping and swimming, tissue samples were collected from mice in each of the three groups on days 1, 3, 5 and 7 after MPTP injection, to carry out immunohisto-chemistry staining of DA neurons in the nigra-striatum. Mice were anaesthetized with 1 ml IP pentobarbital (0.5%). After sternotomy, they were instantly perfused with 0.9% saline through the aorta for 5 min and then with a fixative (4% para-formaldehyde in 0.1 M phosphate buffered saline [PBS], pH 7.1) for 1 h. Immediately after perfusion, the brain was removed from the skull, fixed with 4% paraformaldehyde for 5 h and then washed and soaked in PBS with 30% sucrose at 4 °C until it sank. Using a mouse brain atlas as a guide, the brain was divided into two parts along the median line. The left part of the brain was then cut coronally to test for TH-positive neurons in the substantia nigra and for TH-positive terminals in the striatum. The right part of the brain was cut sagittally to test for TH-positive nerve fibres. All of the sections were cut 10 μm thick in a cryostat at –22 °C. Glass slides were pre-treated with gel, air dried and stored at –20 °C.

IMMUNOHISTOCHEMISTRY STAINING OF DA NEURONS
TH staining
Brain sections were air-dried at room temperature and soaked in 0.1 M PBS for 10

min. After being treated with 99% methanol/1% hydrogen peroxide for 30 min to remove the endogenous peroxidase, sections were washed three times in 0.1 M PBS and then incubated with 10% goat serum for 1 h. The sections were incubated with primary mouse anti-TH monoclonal antibody (diluted 1:3000, Sigma, St Louis, MO, USA) for 24 h at 4 °C and then with biotin-conjugated donkey anti-mouse antibody (diluted 1:500, Sigma) overnight at 4 °C. Finally, the sections were incubated with Cy3-labelled avidin D (diluted 1:1000, Chemicon International, Temecula, CA, USA) for 2 h at 20 °C. After incubations the slides were washed three times in PBS (5 min each time), and were developed for 5 min using avidin–peroxidase complex (Vectastain® ABC kit, Vector Laboratories, Burlingame, CA, USA), as per the manufacturer's instructions, with diaminobenzidine as the chromogen and then evaluated.

Fluoro-Jade C® staining

After being immersed in 1% sodium hydroxide/80% ethanol for 5 min, tissue sections were immersed in 70% ethanol for 2 min followed by 2 min immersion in distilled water. Sections were then incubated with 0.06% liquid potassium permanganate for 10 min on a rocking bed at room temperature and then washed for 2 min in distilled water. The treated sections were incubated in 0.0001% Fluoro-Jade® C (FJC) (Chemicon) for 20 min at 20 °C then washed in distilled water three times (1 min each). Sections were then dehydrated in absolute alcohol for 2 min, cleared with xylene three times (2 min each) and covered with neutral gum and a cover slip.

QUANTIFICATION OF NEURONS AND NEURAL FIBRES

Images and positive cell counts were

captured with an Olympus BX51 fluorescence microscope (Olympus, Tokyo, Japan) using a blue filter (excitation wavelength of 450 – 490 nm).

Counts of immunoreactive neurons were obtained from five serial coronal sections from the substantia nigra pars compacta to the substantia nigra pars reticulate Counts of nerve fibres 200 μm away from the substantia nigra neurons were obtained from five serial sagittal sections in the medial forebrain bundle. A mean count of immunoreactive neurons or nerve fibres per section was determined for each animal by microscopy at ×200 magnification.

ELECTRON MICROSCOPY

After behavioural testing (pole-jumping and swimming), mice from each of the three groups were excessively anaesthetized using 2% pentobarbital 50 mg/kg IP on days 1, 3, 5 and 7 after injection with MPTP. After sternotomy, the mice were then perfused through the left ventricle with 30 ml of 37.0 °C saline and then with 4 °C fixative (described above) for 2 h. Immediately after perfusion, each brain was removed from the skull and the substantia nigra was accurately separated by anatomical microscopy. Tissues were cut into 1 mm³ cubes and then fixed with 2.5% glutaraldehyde/1% osmic acid, then dehydrated with ascending concentrations of 50%, 70% and 90% ethanol followed by 90% acetone, before being embedded in Epon812 (Emicron, Shanghai, China). Ultramicrocuts (600 – 800 μm thick) were stained with uranyl acetate and lead citrate. All sections were examined using a JEM-1200 EX transmission electron microscope (JEOL, Tokyo, Japan) at ×4000 – ×50 000 magnification.

STATISTICAL ANALYSIS

Comparisons between groups were

performed using a one-way analysis of variance followed by the Student–Newman–Keuls test using Origin 7.0 software (OriginLab, Northampton, MA, USA). A probability level (*P*) of < 0.05 was considered to be statistically significant. Results are expressed as mean ± SE.

Results

ANIMALS

Eight mice were included in each of the three groups. For each group two animals each were sacrificed (as described) on days 1, 3, 5 and 7 following MPTP injection, one for analysis by immunohistochemisty and one for analysis by electron microscopy.

BEHAVIOURAL TESTS

There were no ethological differences between the acute and sub-acute PD mode mice treated with MPTP. After short-term treatment with MPTP, mice showed acute abnormal behaviour, such as high-frequency trembling, hypolocomotion, gazing, polypnoea, arched back, stretched hind limbs, unstable gait, tail hyperextension, irritability and piloerection, etc. Some animals showed epilepsia-like manifestations. With increased frequency and duration of MPTP administration, mice showed reduced progressive spontaneous motor activity, trembling, drooling, lassitude, clumsy limb activity and even inactivity, although the acute manifestations were decreased. The MPTP-treated mice frequently fell in a pole-jumping test and scored zero in a swimming test. Drinking, eating and the weight of the mice decreased significantly in the two test groups compared with the control group. Animals in the control group showed normal behaviour for all tests.

TH- AND FJC-IMMUNOREACTIVE NEURONS IN SUBSTANTIA NIGRA

The TH-positive DA neurons and their axons and dendrites showed strong fluorescence and had dense distribution with clear and integrated outlines in the control substantia nigra group (Fig. 1A, 1A'), they were loosely distributed with clear partial and integrated outlines in the acute substantia nigra group (Fig. 1B, 1B'), and they were sparsely distributed without clear or integrated
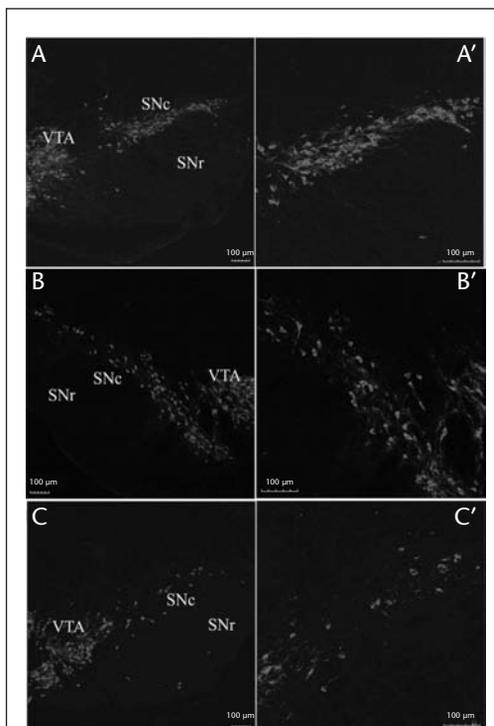
FIGURE 1: Tyrosine hydroxylase (TH) staining in the substantia nigra-striatum, showing TH-positive dopaminergic (DA) neurons and their axons and dendrites in the control group at (A) ×10 and (A') ×20 original magnification; in the acute group at (B) ×10 and (B') ×20 original magnification; and in the sub-acute group at (C) ×10 and (C') ×20 original magnification (SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulate; VTA, ventral tegmental area)

outlines in the sub-acute substantia nigra group (Fig. 1C 1C').

No distribution of FJC-positive neurons or their axons or dendrites was found in the control substantia nigra group (Fig. 2A, 2A'). In the acute group, FJC-positive neurons were distributed in the substantia nigra with regular bright green soma, except for unclear nuclei, and there was no distribution of FJC-positive axons or dendrites, except for a few axonal roots (Fig. 2B, 2B'). Fewer FJC-positive neurons were distributed in the substantia nigra in the sub-acute group, with irregular soma and unclear nuclei compared with the acute group, and there was no distribution of FJC-positive axons or dendrites (Fig. 2C, 2C').
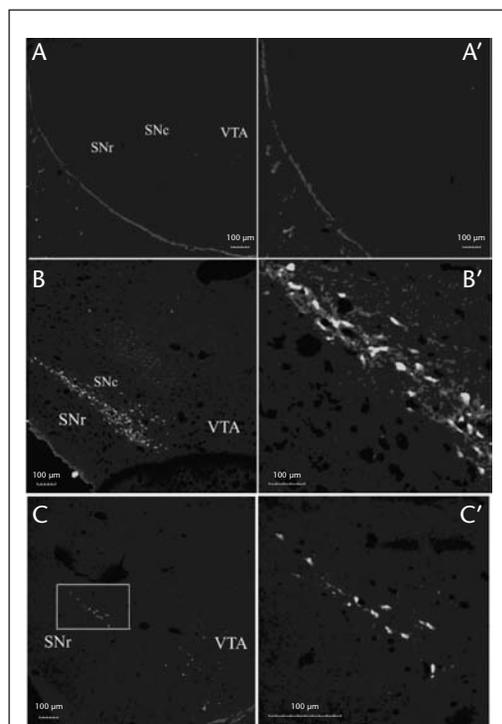
LOSS OF DA NEURONS AND AXONS IN SUBSTANTIA NIGRA

Compared with the control group, the number of DA neurons decreased significantly in both the acute and sub-acute groups ($P < 0.05$), the loss being greater in the acute group than in the sub-acute group, although this difference was not statistically significant (Fig. 3). The number of nerve fibres decreased significantly compared with the control group in both the acute and sub-acute groups ($P < 0.05$), and the loss of nerve fibres was significantly greater in the sub-acute group than in the acute group on days 1, 3, 5 and 7 ($P < 0.05$; Fig. 4).

ELECTRON MICROSCOPY

In the control group, the neural sheath (Fig. 5A; white arrow), and the neuromicrotubes and neurofilaments (Fig. 5A; black arrow) of DA neurons were lined up normally, densely and in order. In the PD model groups, varicosities and a layered neural sheath were evident in the axons of the DA neurons, which are signs of axonal degeneration (Fig. 5B; black arrow). Some axons exhibited dissolution and disruption of the neural sheath and degeneration of the neuromicrotubules and neurofilaments (Fig. 5B; white arrow).


FIGURE 2: Fluoro-Jade® C (FJC) staining in the substantia nigra-striatum, showing FJC-positive neurons and their axons and dendrites in the control group at (A) ×10 and (A') ×20 original magnification; in the acute group at (B) ×10 and (B') ×20 original magnification; and in the sub-acute group at (C) ×10 and (C') ×20 original magnification (SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulate; VTA, ventral tegmental area)

Discussion

Axonal degeneration refers to pathological changes after axonal injury, including axonal varicosities, disruptions, shrinkage
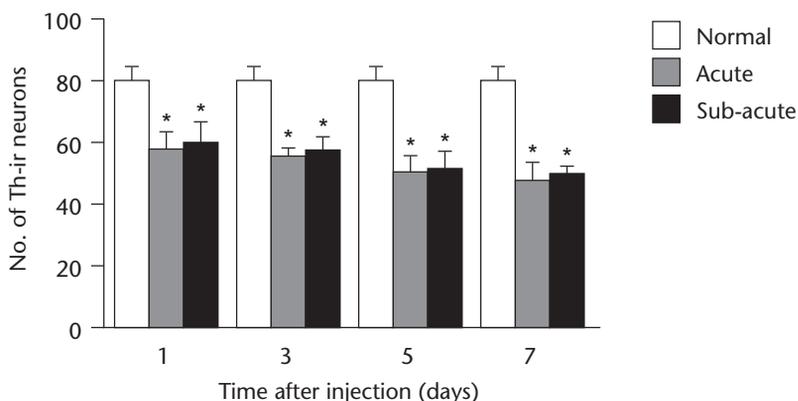
FIGURE 3: The mean ± SE number of tyrosine hydroxylase immunoreactive (Th-ir) dopaminergic (DA) neurons in the control (normal), acute and sub-acute groups, showing a significant decrease in the acute and sub-acute groups compared with the control (*P < 0.05); the difference between the acute and sub-acute groups was not statistically significant
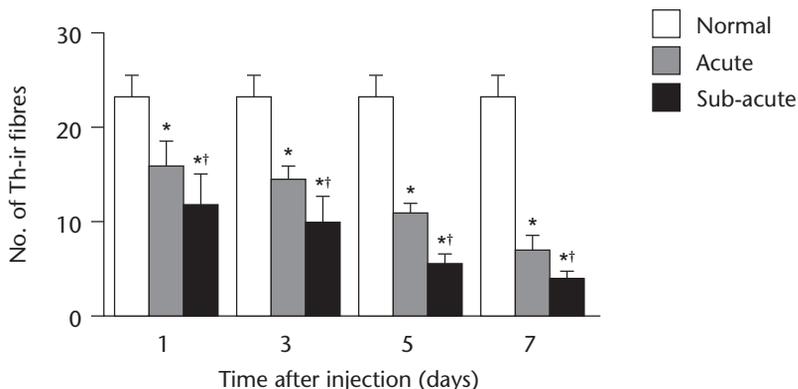
FIGURE 4: The mean ± SE number of tyrosine hydroxylase immunoreactive (Th-ir) nerve fibres in the medial forebrain bundle in the control (normal), acute and sub-acute groups, showing a statistically significant decrease in the acute and sub-acute groups compared with the control (*P < 0.05); and a significantly greater loss of nerve fibres (†P < 0.05) in the sub-acute group versus the acute group

and even axonal cystoskeleton disintegration and axomembrane discontinuity in the severely injured axon.[1,2] After the description of Wallerian degeneration,[4] axonal degeneration was regarded as passive necrosis caused by a decreased supply of nutrition to the perikaryon and, therefore, no therapy or intervention was given to prevent it. In 1989, however, Sajadi *et al.*[5] reported

that distal axons in the Wld[S] mouse did not display axonal degeneration 1 – 2 weeks after the axons had been cut whereas, in the wild-type mouse, there was Wallerian degeneration in the distal axons 2 – 3 days after cutting the axons. Since then, studies on Wld[S] mice have demonstrated that the Wld[S] mutation can postpone axonal degeneration caused by various drugs and gene

FIGURE 5: Electron micrographs of axons in: (A) the control group, showing the neural sheath (white arrow), and neuromicrotubes and neurofilaments (black arrow) of dopaminergic (DA) neurons lined up normally, densely and in order (scale bar 50 nm); and (B) the Parkinson's disease model group, showing varicosities and layered neural sheath in the axons of the DA neurons, which are signs of axonal degeneration (black arrow), and some axons that exhibited dissolution and disruption of the neural sheath and degeneration of the neuromicrotubules and neurofilaments (white arrow) (scale bar 100 nm)

variations.[6,7] This evidence shows that, like apoptosis, axonal degeneration is an active reaction to severe injury of the perikaryon.[3] Studies have shown that axonal degeneration occurs earlier than neuronal apoptosis in many diseases, such as Huntington's disease, that were once thought to be caused solely by neuronal apoptosis.[8] Thus, axonal degeneration is one of the main pathological changes in neural degenerative diseases, suggesting that the prevention of axonal degeneration could be used as a therapeutic strategy to prevent or minimize neural degeneration.

Further studies have shown that, in the development of PD, axons display degeneration (such as varicosities, disruption and shrinkage) that occurs earlier than neuronal apoptosis and protein aggregation (Lewy neuritis) in the nerve fibres, suggesting that axonal degeneration may be one of the causes of neuronal apoptosis.[9,10] In addition, it has been shown that the loss of nerve fibres is higher than the loss of DA neurons in PD, i.e. a loss of 80 – 90% of TH-positive nerve terminals in the striatum was accompanied by a loss of 50 – 60% DA neurons in the substantia nigra.[1] This suggests that dysfunction of the axons and synapses in the DA neurons occurs earlier than in the DA neurons themselves and that not all surviving DA neurons have intact axons and, therefore, cannot function.

The block of axoplasmic transport caused by exogenous or endogenous factors may lead to axonal mutilation.[11] Once the target cells are damaged, axons will irreversibly lose their link with the target cells because axons have no self-regenerative capacity in the central nervous system, and loss of the link with the target cells can only be compensated through gemmation of the remaining neurons.[12] Mutilated axons may shrink after injury and severe shrinkage may induce 'dying-back' degeneration of neurons,[13] suggesting that axonal degeneration may be

one of the causes of apoptosis and that it is impossible to prevent apoptosis without preventing axonal degeneration.

Although mice in the two PD model groups in the present study displayed similar behaviour 1 week after injury, there were significant differences in the pathological changes between the two groups. In the acute group, the loss of substantia nigra DA neurons and the degeneration of their axonal fibres occurred almost simultaneously whereas, in the sub-acute group, a significant loss of TH-positive substantia nigra DA neurons was not evident until 5 days after the injury and some neurons were still surviving on day 7. Thus, injury to the nerve fibres, which may determine the symptoms of the PD animal model, is more evident than loss of neurons and may be consistent with the natural course of PD.

It has gradually been recognized that axonal degeneration plays an important role in PD morbidity, but the role and mechanism of axonal degeneration in PD needs to be clarified for the further study of specialized therapies. Exogenous substances, such as MPTP and rotenone, or endogenous mutant genes, such as α-*syn* or *parkin*, share the same target on the microtubule in reducing microtubule stability.[10] The disaggregated microtubule is more likely to be degraded than the aggregated microtubule,[14] which means that axonal degeneration exhibits microtubule disaggregation early in the axonal degenerative process; hence, if microtubule disaggregation could be inhibited, microtubule disintegration may be delayed. The prevention of axonal degeneration in the early stage after injury may, therefore, be a better therapeutic strategy for preventing the development of PD.[8,12] In the present study, the number of nerve fibres in the microtubules in the medial forebrain bundle decreased significantly in the PD model mice, suggesting that disaggregation and degradation of the axonal microtubules in DA neurons, that may be caused by a variety of factors, and the subsequent block of axoplasmic transport may be the main cause of axonal degeneration and subsequent neuronal degeneration in PD. Previous studies have shown that collapsin response mediator protein-2 (CRMP-2) is involved in the dopamine-induced shrinkage and apoptosis of axons of DA neurons[15] and the latest study shows that phosphorylation of CRMP-2 can cause disaggregation of neuronal microtubules and axonal degeneration.[16] These findings suggest that the phosphorylation of CRMP-2 may be one of the main causes of the axonal degeneration in PD and needs further study.

In conclusion, axonal degeneration, occurring either independently of or earlier than neuronal apoptosis, plays an important role in PD pathogenesis. The development of PD cannot be prevented by the inhibition of neuronal apoptosis alone. Instead, the combined inhibition of neuronal apoptosis and axonal degeneration is needed to optimize the integrity of the substantia nigra-striatum pathway, thereby potentially preventing the fundamental development of PD. Studies of the role and mechanism of axonal degeneration in PD have provided not only a theoretical basis for innovation but also a possible new therapeutic strategy.[7]

Conflicts of interest

The authors had no conflicts of interest to declare in relation to this article.

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Author's address for correspondence
Dr Guo-dong Gao
Department of Neurosurgery and the Institute of Functional Brain Disorders, Fourth Military Medical University, 1 Xinsi Road, Xi'an 710038, China.
E-mail: gaogd2007@163.com