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Downregulation of thioredoxin reductase 1 expression in the substantia nigra pars compacta of Parkinson's disease mice

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Research Highlights

- (1) There is no direct evidence supporting a role for thioredoxin reductase in the onset of Parkinson's disease.
- (2) Because of the susceptibility of nerve cells to oxidative injury and the anti-oxidative effect of thioredoxin reductase, we speculated that the enzyme may be involved in the onset of Parkinson's disease.
- (3) In this study, the expression of thioredoxin reductase 1 in the substantia nigra pars compacta of Parkinson's disease mice was significantly decreased, suggesting that the protein may be associated with disease onset. Therefore, regulating thioredoxin reductase expression may be an effective treatment strategy for Parkinson's disease.

Abstract

Because neurons are susceptible to oxidative damage and thioredoxin reductase 1 is extensively distributed in the central nervous system and has antioxidant properties, we speculated that the enzyme may be involved in the pathogenesis of Parkinson's disease. A Parkinson's disease model was produced by intraperitoneal injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine into C57BL/6 mice. Real-time reverse transcription-PCR, western blot analysis and colorimetric assay showed that the levels of thioredoxin reductase 1 mRNA and protein were decreased, along with a significant reduction in thioredoxin reductase activity, in the midbrain of Parkinson's disease mice compared with normal mice. Immunohistochemical staining revealed that the number of thioredoxin reductase 1-positive neurons in the substantia nigra pars compacta of Parkinson's disease mice was significantly decreased compared with normal mice. These experimental findings suggest that the expression of thioredoxin reductase 1 in the substantia nigra pars compacta of Parkinson's disease mice is significantly decreased, and that the enzyme may be associated with disease onset.

Key Words

neural regeneration; brain injury; neurodegeneration; Parkinson's disease; 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; midbrain; substantia nigra pars compacta; tyrosine hydroxylase; oxidative stress; thioredoxin reductase; grants-supported paper; neuroregeneration

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INTRODUCTION

Parkinson's disease is the second most prevalent neurodegenerative disease globally, affecting 1–2% of the population over 65 years of age^[1]. Its manifestations are tremor, bradykinesia, rigidity and abnormal postural reflexes^[2]. The major pathological hallmarks of Parkinson's disease include the progressive loss of dopaminergic neurons in the substantia nigra pars compacta, a dramatic reduction in striatal dopamine levels and the presence of neuronal proteinaceous aggregates called Lewy bodies^[3-4]. Despite the fact that the etiology of Parkinson's disease has been intensively investigated for several decades, our understanding of the pathogenetic basis of Parkinson's disease is still limited. Accumulating evidence suggests that a variety of factors, including oxidative stress, mitochondrial dysfunction and apoptosis, are involved in the pathogenesis of Parkinson's disease^[5-8]. Among these, oxidative stress is considered the major contributor to the progression of Parkinson's disease.

Molecular oxygen is required for numerous enzymatic reactions and energy production in cells. However, these processes produce reactive oxygen species, such as superoxide anion, hydrogen peroxide and hydroxyl radical^[9]. Excessive production of reactive oxygen species is detrimental to cell membranes and can cause cell death. To protect cells from these toxic reactive oxygen species, antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase, as well as antioxidants such as glutathione and vitamin E, are present in cells to restore redox balance. Brain tissues are particularly susceptible to oxidative damage because of the high levels of polyunsaturated fatty acids in the membranes and the relatively low levels of endogenous antioxidant enzymes^[10]. The elevated levels of oxidative stress in Parkinson's disease manifests as higher levels of lipid peroxidation^[11-13], greater nucleic acid oxidation^[14] and increased iron content in the dopaminergic regions of the brain^[15-16]. Elevated levels of oxidized and nitrated proteins are also observed in the

substantia nigra of Parkinson's disease patients^[17-18]. Concomitantly, decreased levels of antioxidants, such as glutathione peroxidase, glutathione and ceruloplasmin, are observed in Parkinsonian brains^[19-21].

Thioredoxin- and glutathione-dependent systems play important roles in cellular defense against oxidative stress and help to maintain redox homeostasis by regulating thiol-disulfide exchange^[22]. The thioredoxin system consists of thioredoxin, thioredoxin reductase and nicotinamide adenine dinucleotide phosphate. Thioredoxin reductase can reduce, and thereby activate, thioredoxin, which serves as a reducing equivalent and catalyzes many redox reactions. Thioredoxin reductase also has the ability to reduce a wide range of other substrates, such as ribonucleotide reductase, lipoamide, lipoic acid, ascorbate, hydrogen peroxide, lipid hydroperoxides, alloxane and ubiquinone Q10^[23]. In addition to its role as an antioxidant, the thioredoxin system participates in several other cellular processes as well, including cell proliferation, angiogenesis, apoptosis and signaling^[24]. So far, three thioredoxin reductases have been identified in mammals: (i) cytosolic thioredoxin reductase 1 (also known as TrxR1 or Txnrd1), (ii) mitochondrial thioredoxin reductase 3 (also known as TrxR2 or Txnrd2) and (iii) thioredoxin-glutathione reductase (also known as thioredoxin reductase 2 or Txnrd3), which contains an additional N-terminal glutaredoxin domain^[25]. Cytosolic thioredoxin 1 and cytosolic thioredoxin reductase 1 are essential for cell proliferation; they provide reducing equivalents to ribonucleotide reductase, and are thus involved in the maintenance of the deoxynucleotide triphosphate pool^[26].

Thioredoxin reductase is widely expressed and is abundant in the central nervous system. Both thioredoxin reductase and thioredoxin are indispensable for embryonic development in mammals^[27]. Lovell *et al*^[28] showed that thioredoxin reductase and thioredoxin protect against A β -induced neurotoxicity. In addition, other studies revealed

that A β -mediated retinal neurotoxicity involves impairment of the thioredoxin system and decreased thioredoxin reductase activity^[29]. Kudin *et al*^[30] highlighted the important contribution of the thioredoxin-2 system to hydrogen peroxide detoxification in the rat hippocampus. These findings highlight the role of thioredoxin reductase as an important source of reducing equivalents in the brain. Among the three thioredoxin reductase isoforms, thioredoxin reductase 1 is expressed at the highest level in the brain^[31]. However, the role of thioredoxin reductase 1 in Parkinson's disease remains unclear. Therefore, the present study was undertaken to investigate the changes in thioredoxin reductase 1 expression in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson's disease.

RESULTS

Quantitative analysis of experimental animals

Twelve C57BL/6 mice were randomly and equally divided into two groups: normal and MPTP. Mice in the MPTP group were intraperitoneally injected with 20 mg/kg MPTP hydrochloride dissolved in saline, with four injections at 3-hour intervals over a period of 1 day, to produce a model of Parkinson's disease^[32]. Mice in the normal group received an equivalent volume of saline. All 12 mice were involved in the final analysis, without any dropout.

The number of tyrosine hydroxylase-positive neurons was decreased in the substantia nigra pars compacta of MPTP-treated mice

One characteristic of Parkinson's disease is the loss of dopaminergic neurons in the substantia nigra pars compacta^[33]. Tyrosine hydroxylase is the rate-limiting enzyme in dopamine synthesis, and is regarded as a marker of dopaminergic neurons. Seven days after the last MPTP injection, tyrosine hydroxylase expression in the substantia nigra pars compacta was determined by immunohistochemistry. The number of tyrosine hydroxylase-immunoreactive neurons in the MPTP group was significantly lower than in the normal group ($P < 0.05$; Figure 1), indicating successful establishment of the Parkinson's disease model.

MPTP decreased thioredoxin reductase 1 expression and thioredoxin reductase activity in the mouse midbrain

To examine the role of thioredoxin reductase 1 in the MPTP mouse model of Parkinson's disease, western blot analysis was performed to observe the changes in thi-

oredoxin reductase 1 protein expression in the midbrain 7 days after the last injection of MPTP. As shown in Figure 2A, B, the MPTP-treated mice showed a 40% decrease in thioredoxin reductase 1 protein level compared with the normal mice ($P < 0.05$).

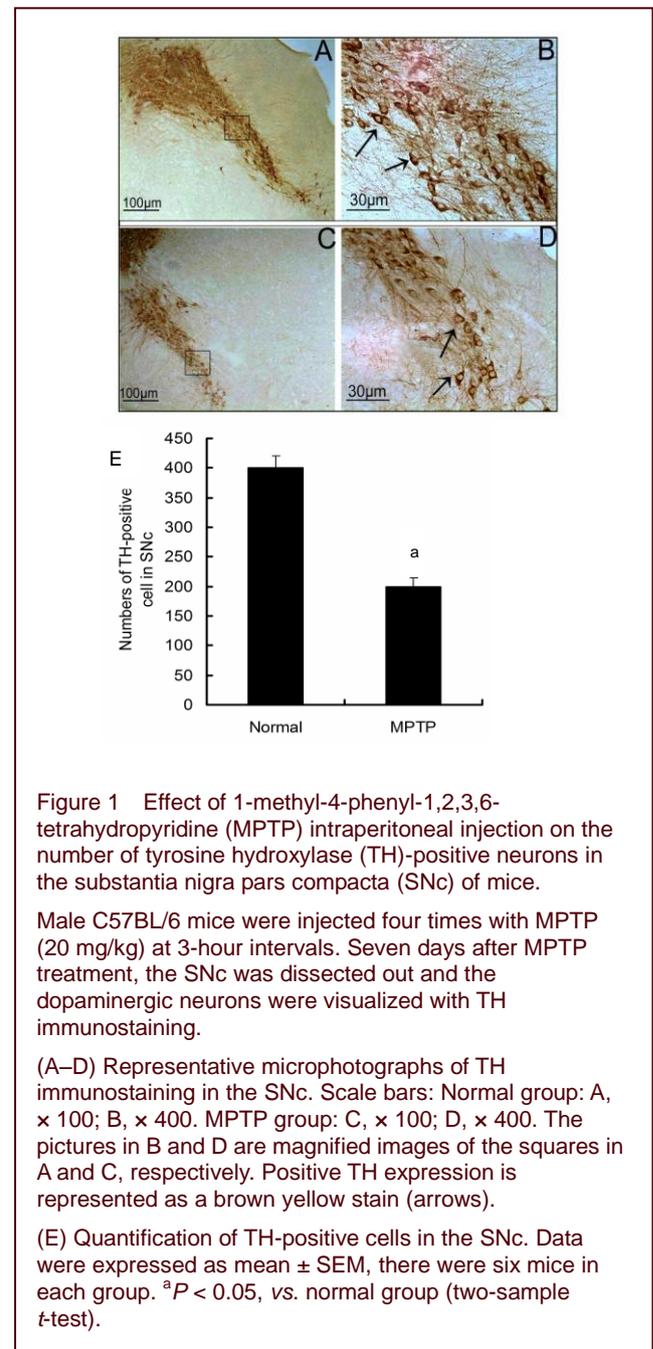


Figure 1 Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) intraperitoneal injection on the number of tyrosine hydroxylase (TH)-positive neurons in the substantia nigra pars compacta (SNc) of mice.

Male C57BL/6 mice were injected four times with MPTP (20 mg/kg) at 3-hour intervals. Seven days after MPTP treatment, the SNc was dissected out and the dopaminergic neurons were visualized with TH immunostaining.

(A–D) Representative microphotographs of TH immunostaining in the SNc. Scale bars: Normal group: A, $\times 100$; B, $\times 400$. MPTP group: C, $\times 100$; D, $\times 400$. The pictures in B and D are magnified images of the squares in A and C, respectively. Positive TH expression is represented as a brown yellow stain (arrows).

(E) Quantification of TH-positive cells in the SNc. Data were expressed as mean \pm SEM, there were six mice in each group. ^a $P < 0.05$, vs. normal group (two-sample *t*-test).

Thioredoxin reductase 1 mRNA expression in the midbrain of mice was measured using real-time reverse transcription-PCR after the mice were injected with MPTP for 7 days. There was a pronounced reduction in thioredoxin reductase 1 mRNA level in MPTP-treated mice compared with normal mice ($P < 0.05$; Figure 2C).

Thioredoxin reductase activity in the midbrain was ana-

lyzed using a colorimetric method. As shown in Figure 2D, the activity of thioredoxin reductase in the MPTP group was significantly reduced by 28% compared with the normal group ($P < 0.05$).

MPTP reduced the number of thioredoxin reductase 1-positive cells in the substantia nigra pars compacta of mice

Finally, we performed immunohistochemistry to assess levels of thioredoxin reductase 1 protein in the substantia nigra pars compacta 7 days following the last MPTP injection. As shown in Figure 3, the number of thioredoxin reductase 1-positive cells in the MPTP group was significantly lower than in the normal group ($P < 0.05$).

DISCUSSION

Parkinson's disease is a progressive neurodegenerative disease that mainly afflicts the elderly. It is characterized by slow degeneration of dopaminergic neurons in the substantia nigra pars compacta, resulting in a decrease in dopamine levels in the striatum. Administration of the toxin MPTP can cause neurochemical, behavioral and histo-

pathological alterations in human and nonhuman primates that are similar to those observed in Parkinsonian patients. Compared with primates, rodents are insensitive to MPTP^[34]. However, C57BL/6 mice were found to be more sensitive to MPTP than other mouse strains^[3]. Therefore, the MPTP-treated C57BL/6 mouse is an excellent model for Parkinson's disease and has been widely used for studies on the disease^[35]. MPTP can be administered by various routes, such as gavage and stereotactic injection, but the most common and reproducible route is systemic administration, including subcutaneous, intravenous, intraperitoneal and intramuscular injection^[36]. In the present study, MPTP was administered by intraperitoneal injection. One characteristic of Parkinson's disease is the loss of dopaminergic neurons in the substantia nigra pars compacta. Tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis, is frequently used to assess dopaminergic neuronal loss in animal models of Parkinson's disease^[37]. After 7 days of MPTP exposure, there is a significant decrease in the number of tyrosine hydroxylase-positive neurons in the midbrain^[38]. In our present study, the successful establishment of the Parkinson's disease model was evaluated after 7 days of MPTP administration.

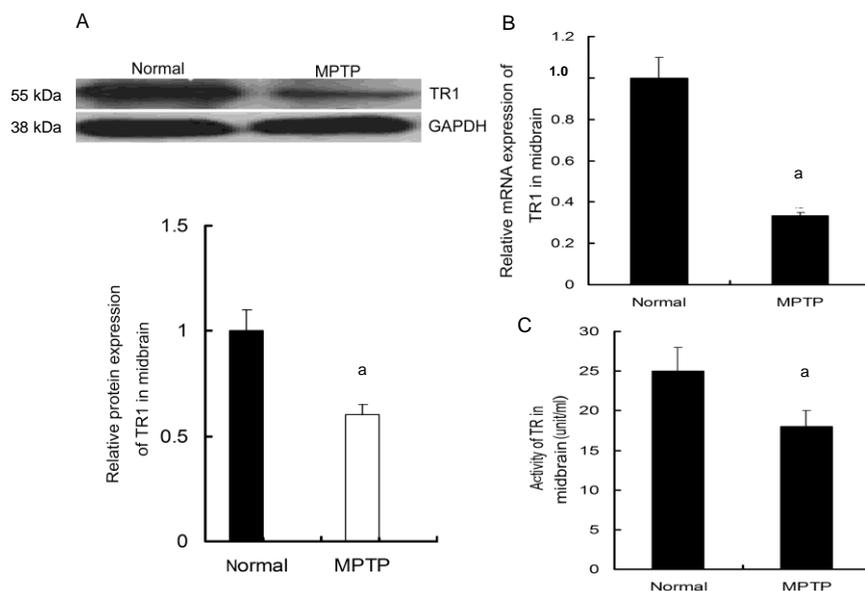


Figure 2 Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) intraperitoneal injection on thioredoxin reductase 1 (TR1) expression and thioredoxin reductase (TR) activity in the midbrain of mice.

Seven days after MPTP treatment, the midbrain of mice was dissected out and TR1 expression levels and TR activity were evaluated. (A) Decreased level of TR1 protein was observed in MPTP-treated mice by western blot analysis. Data are expressed as the ratio of the absorbance of the target gene to that of the GAPDH control. (B) TR1 mRNA level in the midbrain of mice was measured using real-time reverse transcription-PCR. A significant reduction in TR1 mRNA level was found in the MPTP-treated mice. Results are expressed as the ratio of the absorbance of the target gene to that of the GAPDH control. (C) TR activity in the midbrain of the mice was evaluated using a thioredoxin reductase assay kit. MPTP decreased TR activity in the mouse midbrain. Data are expressed as mean \pm SEM, and there were six mice in each group. ^a $P < 0.05$, vs. normal group (two-sample *t*-test).

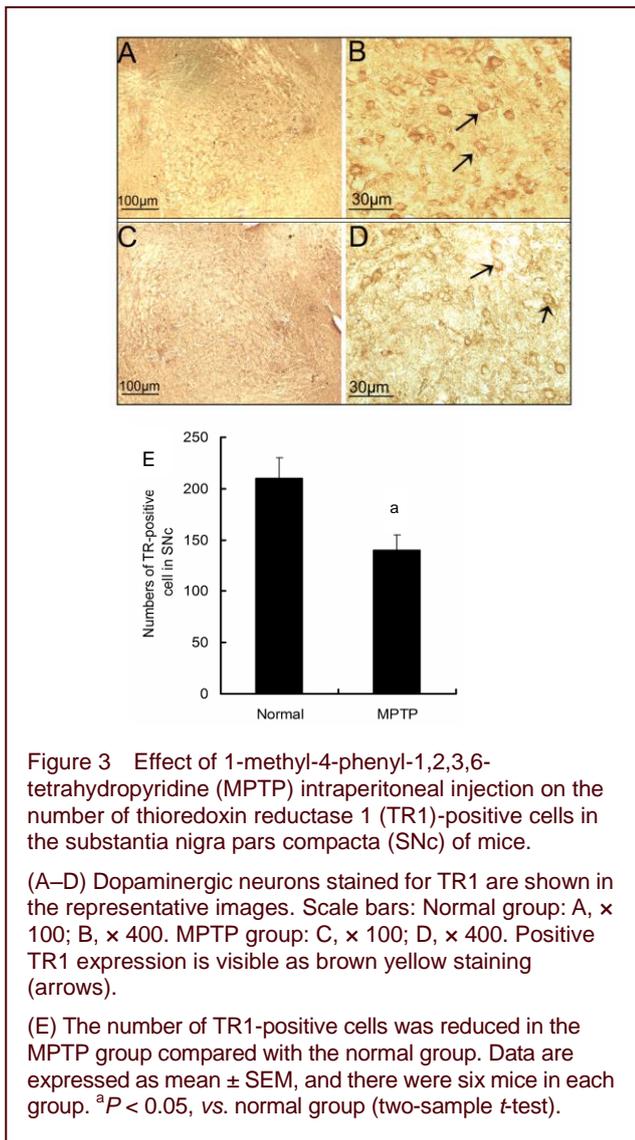


Figure 3 Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) intraperitoneal injection on the number of thioredoxin reductase 1 (TR1)-positive cells in the substantia nigra pars compacta (SNc) of mice.

(A–D) Dopaminergic neurons stained for TR1 are shown in the representative images. Scale bars: Normal group: A, $\times 100$; B, $\times 400$. MPTP group: C, $\times 100$; D, $\times 400$. Positive TR1 expression is visible as brown yellow staining (arrows).

(E) The number of TR1-positive cells was reduced in the MPTP group compared with the normal group. Data are expressed as mean \pm SEM, and there were six mice in each group. ^a $P < 0.05$, vs. normal group (two-sample t -test).

Consistent with previous reports, MPTP administration resulted in a reduction in tyrosine hydroxylase immunoreactivity in the substantia nigra pars compacta, suggesting that dopaminergic neuron loss was present and that the murine Parkinson's model was successfully established.

Oxidative stress is a major pathogenetic feature of neurodegenerative diseases, including Parkinson's disease. Indeed, the brain produces large amounts of reactive oxygen species because of its high metabolic rate and the high content of oxidizable molecules, such as dopamine and neuromelanin, whose metabolism generates reactive oxygen species^[39]. The neurotoxin MPTP is known to cause C57BL/6 mice to develop Parkinsonism. MPTP is a lipophilic protoxin that can rapidly cross the blood-brain barrier following systemic injection. Once it enters the brain, MPTP is converted to 1-methyl-4-phenylpyridine by monoamine oxidase B^[40-41]. The formation

of 1-methyl-4-phenylpyridine is accompanied by increased levels of reactive oxygen species, which attack DNA, protein and membrane lipids, leading to cell death^[42]. Several postmortem studies show that markers for lipid peroxidation, oxidative DNA and protein damage are significantly increased in the substantia nigra pars compacta of Parkinson's disease patients^[6]. It is well known that NADPH oxidase is an important generator of reactive oxygen species in the cerebral vasculature under normal physiological conditions, as well as during ischemia/reoxygenation^[43]. In addition, NADPH oxidase-mediated oxidative stress leads to dopaminergic neuron death in Parkinson's disease^[44-45]. Mutant mice deficient in NADPH oxidase exhibit less dopaminergic neuronal loss in the substantia nigra pars compacta and have lower levels of protein oxidation than do their wild-type littermates after MPTP injection^[46]. Oral treatment with the NADPH oxidase antagonist apocynin can alleviate the pathological changes and symptoms in the MPTP marmoset model of Parkinson's disease^[47]. In contrast to the levels of oxidants, which are elevated, antioxidant components always decline in Parkinson's disease. A reduction in antioxidant enzymes likely contribute to the increased oxidative stress observed in Parkinson's disease. In the substantia nigra pars compacta of the Parkinsonian brain, the balance between the production of free radicals and neutralization by the complex antioxidant system is perturbed. For example, deficiency of superoxide dismutase or glutathione peroxidase, important antioxidant enzymes, has been reported to exacerbate MPTP lesions^[48]. Conversely, overexpression of superoxide dismutase or glutathione peroxidase is regarded as a potential neuroprotective approach in Parkinson's disease models^[49].

Thioredoxin reductase is a crucial antioxidant enzyme. It is a redox-active selenoenzyme, having a selenocysteine residue in its active site^[50]. Its structure and function are similar to glutathione peroxidase and lipoamide dehydrogenase^[51]. Thioredoxin reductase works in conjunction with thioredoxin to form an important cellular disulfide reductase system. It reduces not only the disulfide in oxidized thioredoxin, but also a number of other protein disulfides and a wide spectrum of oxidized low molecular weight compounds^[52]. Most of these substrates are involved in cellular redox regulation. Therefore, thioredoxin reductase plays a key role in the maintenance of the redox balance inside cells. The downregulation of thioredoxin reductase 1 and thioredoxin reductase 3 expression by siRNA treatment has been shown to result in increased sensitivity to oxidative stress, suggesting that these enzymes are important for protection against oxi-

ductive stress^[25]. In addition, inhibition of thioredoxin reductase increases reactive oxygen species levels and induces apoptosis in neuronal cell lines^[53]. It has been shown that hyperbaric oxygen preconditioning alleviates anxiety-like behavior and cognitive impairments by inhibiting neuronal apoptosis and upregulating thioredoxin reductase in stressed rats^[54]. Sulforaphane is able to protect primary cortical neurons against 5-S-cysteinyl-dopamine-induced injury by a mechanism involving the increased expression and activity of phase II enzymes such as glutathione-S-transferase, glutathione reductase, thioredoxin reductase and nicotinamide adenine dinucleotide phosphate oxidoreductase 1^[55]. Recently, it was shown that mitochondrial thioredoxin reductase deficiency potentiates oxidative stress, mitochondrial dysfunction and cell death in dopaminergic cells^[56].

Because of the critical antioxidant and protective roles of thioredoxin reductase in the central nervous system, we explored whether thioredoxin reductase 1 expression is modulated by MPTP *in vivo*. In this study, we observed that thioredoxin reductase 1 mRNA and protein levels were substantially lowered in the midbrains of Parkinson's disease mice. Furthermore, the midbrains of MPTP-treated mice showed a lower activity of thioredoxin reductase, which could contribute to oxidative stress. In addition, we found that thioredoxin reductase 1 protein level was considerably reduced in the substantia nigra pars compacta of MPTP-treated mice compared with normal mice, based on the immunohistochemical results. These findings suggest that MPTP may down-regulate thioredoxin reductase 1 expression in mice. This results in a decrease in the available pool of reduced thioredoxin, thereby leading to a decline in the activities of many antioxidant enzymes that require thioredoxin as an electron donor. The diminished levels of these antioxidant enzymes exacerbate reactive oxygen species accumulation and promote a harmful shift in the redox balance within cells.

In conclusion, the present study suggests that thioredoxin reductase 1 expression is decreased in the substantia nigra pars compacta of the Parkinson's disease mouse model. The decrease in the thioredoxin reductase 1 expression may contribute to the increased oxidative stress and subsequent neurodegeneration observed in the brain of the Parkinsonian mice. Thioredoxin reductase 1 may provide a promising therapeutic target for the development of novel and effective treatment strategies for Parkinson's disease. It should be interesting to examine whether increasing thioredoxin reductase 1 expression can protect against neurotoxin-induced neu-

ronal cell death in Parkinson's disease.

MATERIALS AND METHODS

Design

A randomized, controlled animal experiment.

Time and setting

Experiments were performed at the Institute of Anatomy and Embryology, School of Basic Medical Sciences, Lanzhou University, China, from April 2011 to May 2012.

Materials

Male C57BL/6 mice, 8-week-old, weighing 20–25 g, were provided by Animal Center of Lanzhou University, China (license No. SCXK (Gan) 2009-0004). The animals were housed in groups of four in polycarbonate cages and maintained on a 12-hour light/dark cycle at 22°C. The animals had free access to food and water. Experimental procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China^[57].

Methods

Establishment of MPTP mouse model of Parkinson's disease

Male C57BL/6 mice were randomly divided into two groups, normal ($n = 6$) and MPTP ($n = 6$). Mice in the MPTP group were intraperitoneally injected four times (at 3-hour intervals over a 1-day period) with 20 mg/kg MPTP hydrochloride (M-0896, Sigma-Aldrich, St. Louis, MO, USA) dissolved in saline^[32]. Mice in the normal group received 20 mg/kg saline. The successful establishment of the Parkinson's disease mouse model was determined by evaluating tyrosine hydroxylase expression in the substantia nigra pars compacta.

Western blot analysis for thioredoxin reductase 1 protein expression in the midbrain of MPTP-treated mice

Seven days after MPTP treatment, the mice were sacrificed by decapitation. The whole brains were immediately removed and placed on a plate over crushed ice. The midbrain was then dissected, frozen in liquid nitrogen and preserved at -80°C for subsequent western blot analysis. Proteins were fractionated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Membranes containing fractionated cytosolic proteins were blotted with anti-thioredoxin reductase 1 antibody (mouse monoclonal

antibody, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-GAPDH antibody (mouse monoclonal antibody, 1:5 000; Millipore Corporation, Billerica, MA, USA). Membranes were then blotted with horseradish peroxidase conjugated secondary antibody (goat anti-mouse, 1:5 000; Jackson, Mercer, PA, USA) and the immunoreactive protein bands were visualized with enhanced chemiluminescence (Millipore Corporation). Densitometry analysis was performed using Image J software (NIH, Bethesda, MD, USA). Data are expressed as the ratio of the absorbance of the target gene to that of the GAPDH control.

Quantitative real-time reverse transcription-PCR detection of thioredoxin reductase 1 mRNA expression in the midbrain of MPTP-treated mice

After removal of the brain, the tissue samples were flushed briefly with ice-cold saline and blotted with filter paper to remove excess liquid. The midbrain was excised, frozen in liquid nitrogen, and stored at -80°C . Total RNA from the homogenized tissue samples was isolated using the RNAiso Plus RNA isolation kit (Takara, Dalian, Liaoning Province, China). Primer sequences for real-time reverse transcription-PCR are as follows:

Gene	Sequence (5'-3')
Thioredoxin reductase 1	Forward: AGG AAC TCT GTC AGG ACA GCC AGT A
	Reverse: GCC AGC ATG TTC ACG GTC A
GAPDH	Forward: TGT GTC CGT CGT GGA TCT GA
	Reverse: TTG CTG TTG AAG TCG CAG GAG

GAPDH: Glyceraldehyde phosphate dehydrogenase.

RNA quantity was measured by spectrophotometric quantification (NanoDrop 2000, Peqlab, Erlangen, Germany). Total RNA (1.0 μg) was transcribed to cDNA using Reverse Transcriptase M-MLV and oligo dT primer (Takara, Dalian, China). Quantitative real-time PCR was performed with SYBR premix Ex Taq (Takara) using the Rotorgene 3 000 system (Corbett, Sydney, Australia). The PCR conditions were: 95°C for 10 seconds, followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. Results are expressed as the absorbance of the target gene normalized to that of the GAPDH control.

Colorimetric assay for thioredoxin reductase activity in the midbrain of MPTP-treated mice

Thioredoxin reductase activity was determined using the Thioredoxin Reductase Assay Kit (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, 7 days after MPTP treatment, the mice were sacrificed by decapitation and brains were rapidly removed and placed on an ice-cold platform and midbrains were dissected out.

The midbrain was lysed with a buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl, 1% Triton X-100, 1% sodium dodecyl sulfate, 1% deoxycholate, 1 mmol/L NaF and 1 mmol/L EDTA, and protease inhibitors. After centrifugation at $14\ 000 \times g$ for 10 minutes at 4°C , protein concentrations of supernatants were determined using the Bradford method, and protein was then incubated in 100 mmol/L of potassium phosphate with 10 mmol/L EDTA and 0.24 mmol/L NADPH with and without a thioredoxin reductase inhibitor. The reaction was started by adding 5,5'-dithiobis(2-nitrobenzoic) acid and monitored spectrophotometrically at 412 nm. Enzymatic activity was quantified using the following equation: $\text{Unit/mL} = A_{412\text{nm}}/\text{minute (thioredoxin reductase)} \times \text{dil} \times \text{vol}/\text{enzvol}$ (dil = sample dilution factor; vol = volume of reaction in mL; enzvol = volume of enzyme in mL).

Immunohistochemical staining for tyrosine hydroxylase activity and thioredoxin reductase 1 expression in the substantia nigra pars compacta of MPTP-treated mice

Immunohistochemistry on brain tissues was performed as previously described^[58] with minor modifications. Mice were subject to cardiac perfusion with 0.9% normal saline followed by 0.1 mol/L phosphate buffer containing 4% paraformaldehyde. Brains were then removed, and post-fixed in 4% paraformaldehyde overnight, then dehydrated sequentially in 20% and 30% sucrose solutions in 0.1 mol/L phosphate buffer (pH 7.4) until they sank. Coronal sections throughout the substantia nigra were cut on a freezing microtome (Jung Histocut, Model 820-II, Leica, Germany) at a thickness of 30 μm at $-2.54\ \text{mm}$ to $-3.88\ \text{mm}$ from the bregma (according to the atlas of the mouse brain) and stored at -20°C in cryoprotectant solution. Three sections were selected at -2.92 , -3.16 and $-3.40\ \text{mm}$ from the bregma for immunohistochemical staining. In brief, sections were incubated with 0.3% H_2O_2 for 30 minutes and placed in blocking buffer (10% goat serum, 0.3% Triton X-100 in 0.01 mol/L PBS, pH 7.2) for 10 minutes at room temperature followed by incubation with rabbit anti-tyrosine hydroxylase polyclonal antibody (Abcam, 1:300) or mouse anti-thioredoxin reductase 1 monoclonal antibody (Santa Cruz Biotechnology, 1:200) at 4°C overnight. The tyrosine hydroxylase-positive and thioredoxin reductase1-positive cells were identified by reaction with goat biotinylated polyclonal anti-rabbit antibody and goat biotinylated polyclonal anti-mouse antibody (1:200, 1 hour at 37°C ; Millipore Corporation), respectively, in addition to streptavidin-biotin-peroxidase (1:200, 1 hour at 37°C ; Millipore Corporation). Immunoreactivity was visualized with 0.05% 3,3'-diaminobenzidine as chromagen. The tyrosine hydroxylase- and thioredoxin reduc-

tase 1-positive cells were counted on an optical microscope (Motic, Xiamen, China).

Statistical analysis

All data were expressed as mean \pm SEM and statistical analysis was performed using SPSS 17.0 software (SPSS, Chicago, IL, USA). Two-sample *t*-test was used to evaluate differences between MPTP and normal groups. A value of *P* < 0.05 was considered statistically significant.

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