Therapeutic Effects of Multifunctional Tetramethylpyrazine Nitrone on Models of Parkinson's Disease *in Vitro* and *in Vivo*

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Received September 23, 2013; accepted November 18, 2013; advance publication released online December 3, 2013

Parkinson's disease (PD) is the second most common neurodegenerative disease. Although the etiology of PD is not completely understood, it is well-documented that oxidative stress and Ca^{2+}-mediated cellular damage play important roles in the progression of PD. 2-[[(1,1-Dimethylethyl)oxidoimino]-methyl]-3,5,6-trimethylpyrazine (TBN), a novel nitrone derivative of tetramethylpyrazine, has shown significant therapeutic effects in stroke models due to its multiple functions, including calcium overload blockade and free radical-scavenging. In this study, we investigated the neuroprotective and neurorescue effects of TBN on various *in vitro* and *in vivo* models of PD and explored its possible mechanisms of action. The results show that TBN exerted significant neuroprotection on 1-methyl-4-phenylpyridinium (MPP^+)-induced damage in SH-SY5Y cells and primary dopaminergic neurons, as well as on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neuron loss in zebrafish (TBN and MPTP were added simultaneously into the fish embryo medium and the treatment period was 48 h). In the MPTP-induced mouse and 6-hydroxydopamine (6-OHDA)-induced rat PD models, TBN administrated orally twice daily for 14 d (3 d post-MPTP lesion in mice and 7 d post-6-OHDA lesion in rats) exhibited remarkable neurorescue effects to increase the number of dopaminergic neurons. In addition, TBN improved apomorphine-induced rotational behavior in the 6-OHDA-lesioned PD rats. TBN suppressed the MPP^+-induced intracellular reactive oxygen species (ROS) in SH-SY5Y cells, increased the superoxide dismutase (SOD) activity and glutathione (GSH) concentration in the substantial nigra of MPTP-treated mice. These data indicate that TBN protects and rescues dopaminergic neurons from MPP^+ and MPTP/6-OHDA-induced damage by reducing ROS and increasing cellular antioxidative defense capability.

Key words Parkinson's disease; oxidative stress; tetramethylpyrazine nitrone; 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP); 6-hydroxydopamine (6-OHDA)

Parkinson's disease (PD) is one of the most common neurodegenerative diseases today, affecting 4–6 million people worldwide. Current therapies for PD mainly provide symptomatic improvement by replacing neurotransmitters or controlling their metabolism to restore their imbalance.[1)] L-Dopa is still the most effective symptomatic treatment for PD. Although dopamine replacement may alleviate the symptoms of the disease, it does not, however, slow down or stop the progression of neuronal degeneration. Thus far, there is no disease-modifying neuroprotective or neurorestorative therapy available.

Despite many years of intensive research, the causes of PD are still not completely understood. While there is no definitive answer, it is now widely accepted that there is no single "cause" that triggers the disease. Instead, PD likely results from a confluence of genetic and environmental factors. The hallmark of PD is the progressive degeneration of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc).[2,3)] As the DA cells die, less dopamine is produced and transported to the striatum, where the brain coordinates movement. After approximately 50–80% of DA cell dies, patients start to exhibit the classical symptoms of PD, including bradykinesia, postural reflex impairment, resting tremor and rigidity.[2,3)]

The exact mechanism by which DA cells die in PD is not known; however, increasing evidence suggests that oxidative stress induced by reactive oxygen species (ROS) and reactive nitrogen species (RNS) is involved in the progression of dopaminergic neurodegeneration.[4,5)] The brain is particularly vulnerable to oxidative damage due to its high demand for oxygen and its abundance of highly oxidizable substrates, and in SNpc, such as dopamine. Thus, reducing oxidative damage to DA cells could be an effective PD treatment.

Broadly speaking, there are two strategies to reduce oxidative damage: one is to inhibit the production of ROS, and the other to remove existing ROS. DA cells produce excess ROS *via* different mechanisms, including calcium overflow and dopamine oxidation. Calcium influx through L-type calcium channels, especially the $Ca_V1.3$ subunit, to DA cells was reported to result in constant production of free radicals, causing DA cell death.[6)] DA neurons oxidize dopamine using monoamine oxidase, a reaction known to cause production of superoxide and hydrogen peroxide.[7)] Consequently, DA neurons are in a perpetual state of oxidative stress, eventually leading to cell injury and death.

In addition to reducing oxidative damage, reducing neuroinflammation in the brain is also important for effective treatment of PD. Increasing evidence suggests that neuroinflammation contributes to the cascade leading to the progressive neuronal damage in PD.[8)] Up-regulation of pro-inflammatory gene expression and an increase in activated microglia were

The authors declare no conflict of interest.

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observed in 6-hydroxydopamine (6-OHDA)-induced PD models.[9,10]

It is becoming clear that the etiology of PD is complex and that DA cell injury and death are caused by a variety of factors, including oxidative damage and neuroinflammation.

Thus, from a therapeutic point of view, an agent or a combination of agents with different mechanisms of action is needed. In fact, multifunctional drugs that target multiple pathological pathways of PD have been reported previously.[11]

Chuanxiong (*Ligusticum wallichii* Franchat), a traditional Chinese medicinal herb, has been used extensively in Asian countries for hundreds of years to treat heart, kidney, and brain diseases, *etc*.[12] Tetramethylpyrazine (TMP, Fig. 1) is an alkaloid extracted from Chuanxiong. Previous studies have shown that TMP possesses various pharmacological activities, including anti-inflammation,[13,14] calcium antagonism,[15] and free radical scavenging.[16,17] Systematic administration of TMP has been reported to protect against ischemia and spinal cord injury-induced neuronal loss by inhibiting inflammation *in vivo*.[13,14] TMP inhibited inflammatory events *in vivo* possibly by reducing inflammatory cell activation and proinflammatory mediator production.[18,19] Moreover, the potential therapeutic efficacy of TMP against neurodegenerative diseases, such as Alzheimer's disease (AD) and PD, has also been reported. For instance, TMP attenuated D-galactose-induced impairment of learning and memory performance in the rodent model. It also protected kainic acid (KA)-caused massive neuronal cell death in rat brains.[12,20] TMP reduced the L-dopa induced brain oxidative damage in PD rats.[21]

Nitrones were developed as free radical-trapping agents in free radical chemistry and have been tested as therapeutic agents for neural and systemic dysfunctions including atherosclerosis, septicemia, stroke, and AD.[22] *N-tert*-Butyl-α-phenylnitrone (PBN) was shown to reduce brain infarction in transient and permanent rat MCAO models.[23,24] NXY-059, another nitrone, showed positive results when evaluated in various animal stroke models but failed its second phase-3 clinical trial.[25] One reason why NXY-059 failed could be its difficulty in penetrating the blood–brain barrier (BBB). It is well known that negatively charged compounds cannot readily cross the BBB, and NXY-059 has two sodium sulfonate moieties.

We have designed and synthesized a series of novel TMP derivatives, one of which is 2-[[(1,1-dimethylethyl)-oxidoimino]-methyl]-3,5,6-trimethylpyrazine (TBN, Fig. 1). TBN is a TMP derivative armed with a powerful free radical-scavenging nitrone moiety. We have previously reported that TBN has potent free radical-scavenging activity against some of the most damaging radicals, including hydroxyl ($\cdot$OH), superoxide ($\cdot O_2^-$) and peroxynitrite ($ONOO^-$).[26,27] TBN showed remarkable activity protecting neuronal cells from oxidative injury *in vitro* and protected rats from ischemic stroke damage. TBN acts through multiple functions, two of which are blocking Ca^{2+} overload and neutralizing free radicals.[26,27]

The aims of this work are to evaluate the neuroprotective and neurorescue effect of TBN in different models of PD and explore its possible mechanisms of action.

MATERIALS AND METHODS

Reagents 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 1-methyl-4-phenyl-pyridinium ion (MPP^+), 6-hydroxydopamine bromide (6-OHDA) and *R*-(−)-deprenyl (selegiline) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Dulbecco's modified Eagle's medium (DMEM) and basal modified Eagle's medium, heat-inactivated horse serum, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco Invitrogen (Carlsbad, CA, U.S.A.). The fluorescent probes 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DCF-DA diacetate) were purchased from Molecular Probes (Eugene, OR, U.S.A.). The antibody against tyrosine hydroxylase (TH) was purchased from Millipore (Billerica, MA, U.S.A.). TBN was synthesized and purified in our laboratory.[26,28] All other reagents were purchased from Sigma-Aldrich unless stated otherwise.

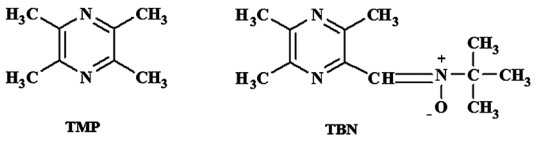

Fig. 1. Structure of TMP and TBN

***In Vitro* Model of PD. SH-SY5Y Cells Culture** Human neuroblastoma SH-SY5Y cells purchased from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.) were grown in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C under a humidified atmosphere of 5% CO_2. The medium was changed every other day. All experiments were performed 48 h after cells were seeded.

Assessment of Cell Viability by 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium Bromide (MTT) Assay MTT is a tetrazolium salt that can be reduced to purple formazan by living cells. Briefly, SH-SY5Y cells (2×10^4 cells/100 μL/well seeded on a 96 well-plate) were incubated with different concentrations of TBN (50, 150, or 500 μM), respectively, for 12 h. MPP^+ (2 mM) was then added, and the cells were incubated for a further 24 h. Finally, 10 μL of MTT (10 mg/mL) in PBS was added, and the cells were incubated for another 4 h at 37°C. The medium was then discarded and 100 μL dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan. The absorbance was measured at 570 nm on a microplate reader (Spectra MAX 340, Molecular Devices Co., CA, U.S.A.). Cell viability was expressed as a percentage of the value of the cells without MPP^+ treatment.

Determination of Intracellular ROS Production SH-SY5Y cells were seeded at 2×10^4 cells/well in 96-well plates and were treated with various concentrations of TBN (50, 150, or 500 μM) or vitamin C (Vit-C, 100 μM), respectively, for 12 h. MPP^+ (2 mM) was then added, and the cells were incubated for another 24 h. Cells pretreated with Vit-C were used as positive control. Cells were then washed with phosphate buffered saline (PBS), and were incubated with 20 μM DCF-DA for 1 h. Fluorescence was measured on a microplate reader (Spectra MAX 340) at an excitation wavelength of 495 nm and an emission wavelength of 515 nm. Fluorescence images were also captured using a fluorescence microscope.

Primary Culture of Midbrain Dopaminergic Neurons and Immunostaining with Antibody against Tyrosine Hydroxylase (TH) The ventral portion of the midbrain, rich in dopaminergic neurons, was dissected from 14-d old Sprague-Dawley (SD) rat embryos into a dish with HBSS (NaCl 8.00 g/L, KCl 0.40 g/L, glucose 1.00 g/L, $Na_2HPO_4\cdot12H_2O$

0.12 g/L, KH_2PO_4 0.06 g/L, pH 7.4). All midbrain isolated was dissociated by trypsinization at 37°C for 10 min. The cells were re-suspended and maintained in a humidified incubator at 37°C under a humidified atmosphere of 5% CO_2. On day 5, the culture medium was discarded, and cells were pre-incubated with fresh medium containing TBN for 2 h. MPP^+ (2 μM) was then added, and the cells were incubated for 48 h. The cells were fixed with 1% paraformaldehyde in PBS, and cellular immunostaining with antibody against tyrosine hydroxylase (TH) was conducted as described.[29]

MPTP-Induced Dopaminergic Neuron Loss in Zebrafish Zebrafish is widely used to investigate neural development for various neurodegenerative diseases.[30] We used the wild type AB strain of zebrafish in this study. Embryos were collected after natural spawning, staged according to standard criteria, and raised at 28.5°C in embryo medium (13.7 mM NaCl, 540 mM KCl, 25 mM Na_2HPO_4, 44 mM KH_2PO, 300 mM $CaCl_2$, 100 mM $MgSO_4$, 420 mM $NaHCO_3$, pH 7.4). Healthy zebrafish embryos were picked and dechorionated manually at 1 d post fertilization (dpf), and were then distributed into a 12-well plate with 20 fish embryos in each well. MPTP at 200 mM and TBN, TMP or positive control (selegiline, 100 μM) were added to the 1 dpf zebrafish embryos, and the embryos were incubated for 48 h. The neuroprotective effect of TBN was analyzed by whole-mount immunostaining performed as described previously.[31]

MPTP-Induced PD Model in Mice. Animals and Treatment Male C57BL/6 mice (20±2 g body weight, 6–8 weeks of age) purchased from the Animal Center of Guangdong Province were allowed to have free access to food and water placed under a 12 h light/dark cycle with *ad libitum*. Mice were allowed 7 d to acclimate before any treatment. MPTP (30 mg/kg/day) was administered intraperitoneally (i.p.) daily for 5 consecutive days to induce experimental Parkinsonism. In order to allow for the full conversion of MPTP to its active metabolite MPP^+, a further 3 d of resting period was allowed.[32] On the 8th day, TBN, selegiline (10 mg/kg), isradipine (2.5 mg/kg) or equal volume of saline was administered, orally twice per day, for 14 d. All animal studies were conducted according to guidelines of the experimental animal care and use committee of Jinan University. The experimental protocols were approved by the Ethics Committee for Animal Experiments of Jinan University.

Tissue Processing and Tyrosine Hydroxylase (TH) Immunohistochemistry Twenty four hours after the last dose of drug administration, 6 animals in each group were anesthetized by i.p. administration of 400 mg/kg chloral hydrate (10%, w/v, dissolved in distilled water), and were perfused intracardially with 10 mL PBS (0.1 mmol/L, pH 7.4) followed by 50 mL of 4% paraformaldehyde (PFA) in PBS. Then, the

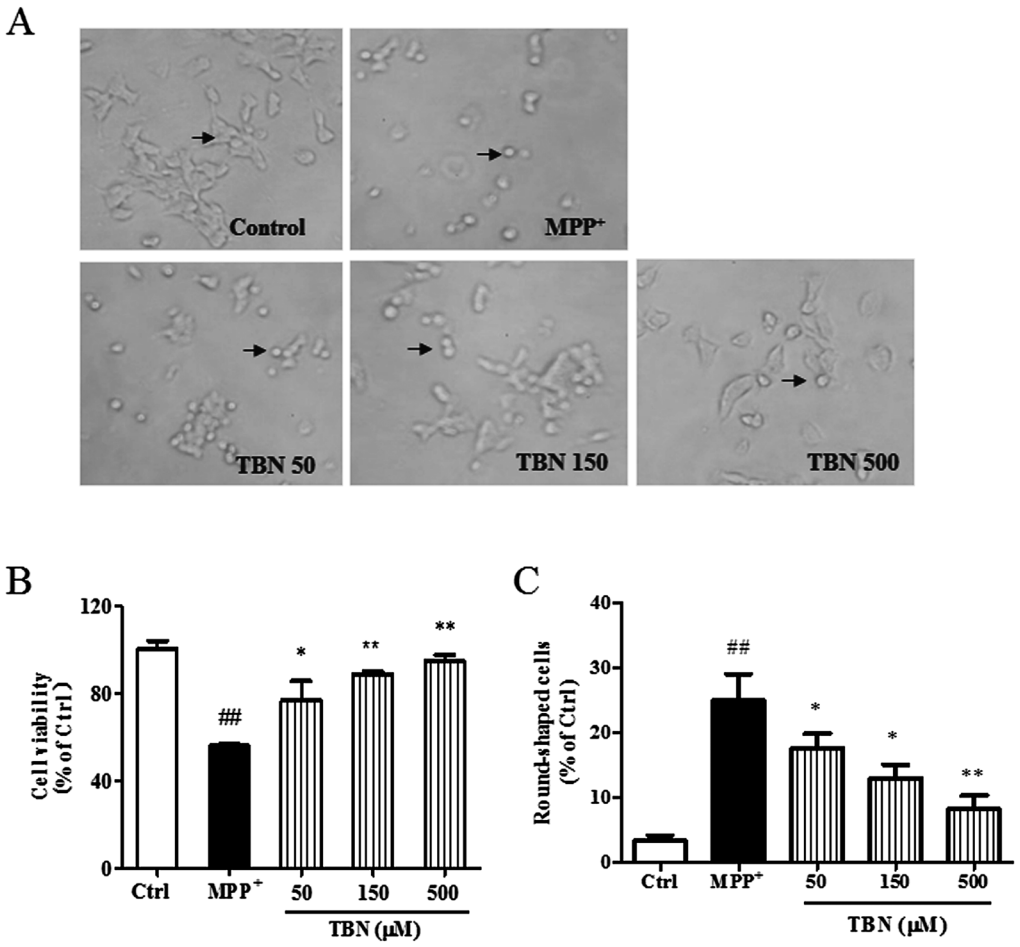

Fig. 2. Cytoprotection of TBN on MPP^+-Induced Toxicity on SH-SY5Y

(A) TBN reversed the morphological alternation induced by MPP^+. Arrow showed the change of cell morphology. (B) Cell viability was measured by the MTT assay and the results are expressed as a percentage of the control group. (C) Quantification of round-sharped cells (average of 6 fields/group). Data were from three independently experiments. $^{\#\#}p<0.01$ *versus* control group (Ctrl); $^*p<0.05$ and $^{**}p<0.01$ *versus* MPP^+ group.

brains were collected and were post-fixed in 4% PFA for another 24 h at 4°C. Samples were embedded in paraffin and were cut into 6 μm coronal sections encompassing the entire SNpc. Another 6 animals in each group were sacrificed by a lethal dose of chloral hydrate. Tissues of substantial nigra were dissected rapidly at 0°C.[33,34] The samples were frozen in liquid nitrogen and were stored at −80°C until processed for further biochemical analysis.

As the decrease of TH-positive neurons in response to MPTP treatment is the most prominent at medial levels of the SNpc,[35] we selected the sections from the area encompassed between −3.08 and −3.20 from bregma to perform the immunohistochemisty analysis. Immunohistochemistry of brain slices and quantification of TH-positive neurons was performed as previously described.[36,37]

Measurement of Superoxide Dismutase (SOD) and Glutathione (GSH) Levels in the Substantial Nigra The substantial nigra tissues were homogenized on ice, and were centrifuged at 10000×g for 10 min at 4°C. The amount of total protein in the supernatant was determined using the BCA protein assay kit (Pierce). SOD activity was detected by the Total Superoxide Dismutase Assay Kit, and GSH using GSH and GSSG Assay Kits (Beyotime Institute of Biotechnology, Beijing, China) according to procedures previously reported.[38]

Unilateral 6-OHDA-Lesioned Model of PD in Rats. Animals Adult male Sprague-Dawley rats weighing 250±25 g were used. Rats were housed, 2 per cage, in a temperature and humidity controlled room, maintained on a 12 h light/dark cycle. The animals were allowed to have free access to food and water.

Surgical Procedures One hundred rats were anesthetized with chloral hydrate (350 mg/kg, i.p.) and were placed

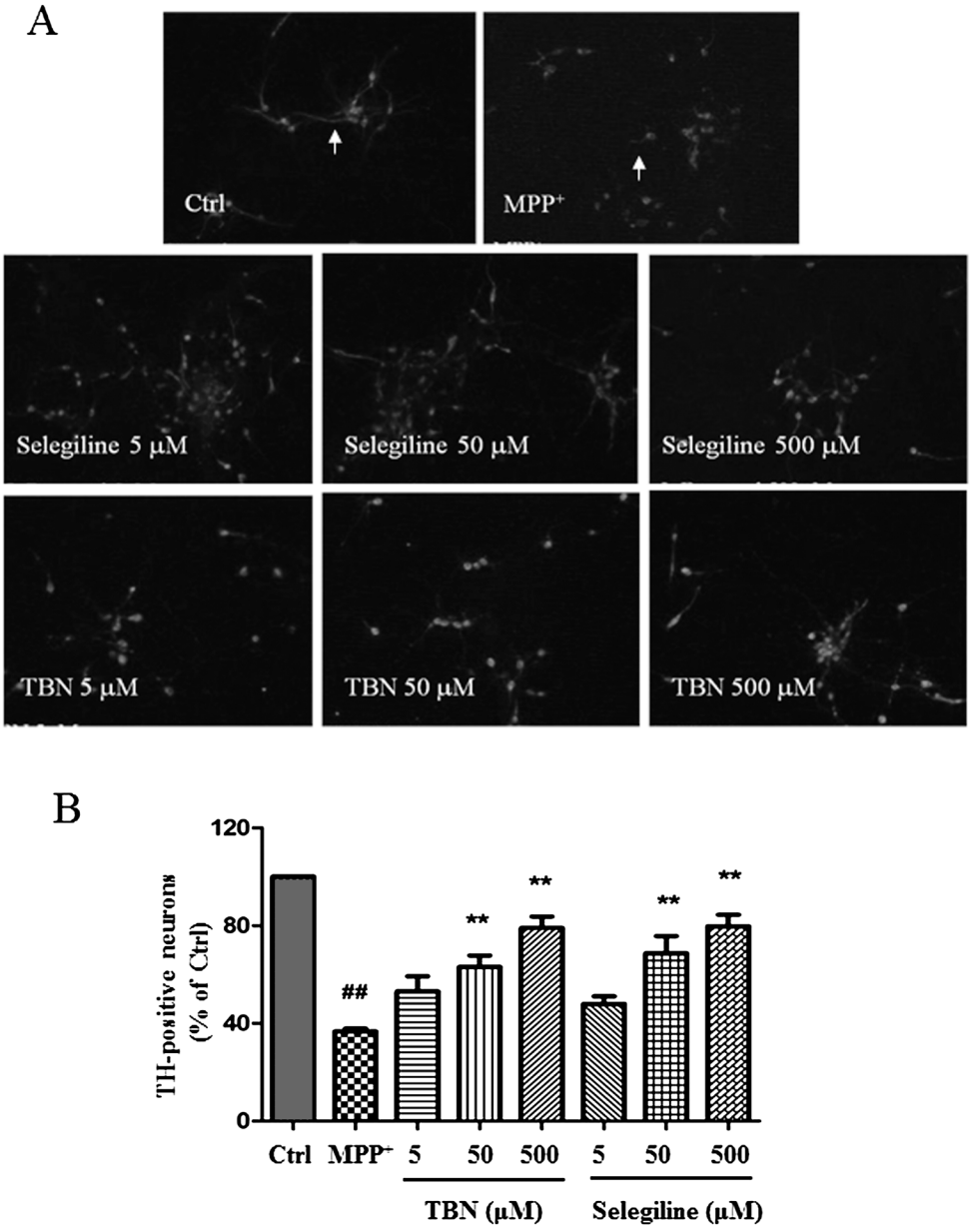

Fig. 3. Neuroprotective Effect of TBN on MPP$^+$-Induced Damage on Cultured Midbrain Dopaminergic Neurons

(A) The representative photographic images of dopaminergic (TH-positive) neurons in the substantial nigra. Arrows showed that the neurites became shortened or disappeared after exposure to MPP$^+$. (B) The number of dopaminergic (TH-positive) neurons per field was quantified by immunohistochemistry and fluorescence microscopy. Data are collected from 20 fields/group (1–2 cultures/group), and are expressed as percentage of the Ctrl group. $^{\#\#}p<0.01$ *versus* Ctrl group; $^{**}p<0.01$ *versus* MPP$^+$ group.

in a stereotaxic instrument (RWD Life Science, China). Eight micrograms of 6-OHDA (4 μL in saline containing 0.2 mg/mL ascorbic acid) was administered into the left substantia nigra pars compacta (SNpc, AP: 5.2 mm; L: 1.8 mm; DV: 8.2 mm) and ventral tegmental area (VTA, AP: 4.6 mm; L: 0.9 mm; DV: 8.2 mm) with a 28-gauge Hamilton syringe, respectively. The administration was conducted at a rate of 0.3 mL/min and the needle was left in place for another 10 min after the administration before slowly drawn back. Sham-operated rats received equal volume of saline instead of 6-OHDA. All animals were given penicillin (i.p.) to prevent postsurgical infection.

Apomorphine-Induced Rotation and Drug Treatment Apomorphine-induced rotation was tested at 1 and 3 weeks post-lesion. Apomorphine was administered s.c. at a dose of 0.5 mg/kg and rotation was monitored for 5 min using the same experimental set up as for apomorphine-induced rotation. Full 360° turns in the direction contralateral to the lesion were counted. Results were expressed as contralateral net turns/min. All behavioral analysis was performed by an observer blinded to the drug administration. Only the rats showing rotational scores $\geq$7 turns/min at one week post-lesion were considered successful for the model of PD, and approximately 50–60% of rats were selected for further drug treatment. On the 8th day post-lesion, TBN, L-dopa (25 mg/kg), selegiline (10 mg/kg) or equal volume of saline was administered orally twice a day for 14 d.

Tissue Processing and Quantitative Immunohistochemistry At 3 weeks post 6-OHDA lesion, all animals were anesthetized with chloral hydrate (350 mg/kg, i.p.), perfused intracardially with 100 mL PBS (0.1 mmol/L, pH 7.4) followed by 250 mL 4% PFA. After surgical removal, brain tissues were collected and post-fixed in 4% PFA for another 24 h at 4°C. Samples were embedded in paraffin and were cut into 6 μm coronal sections encompassing the entire SNpc (at -4.8 and -5.3 mm to the bregma). The procedure of immunohistochemistry and quantification of TH-positive neurons were conducted as described previously.[36,37]

Statistical Analysis Data were expressed as the mean$\pm$S.D. Statistical analysis was performed using one-way ANOVA by means of a Dunnett multiple comparisons test. A probability for all experimental values $p<0.05$ was accepted as an indication of statistically significant difference.

RESULTS

TBN Protects SH-SY5Y Cells against MPP^+-Induced Cytotoxicity As shown in Fig. 2A, exposure to MPP^+ at the concentration of 2 mM for 24 h significantly decreased the

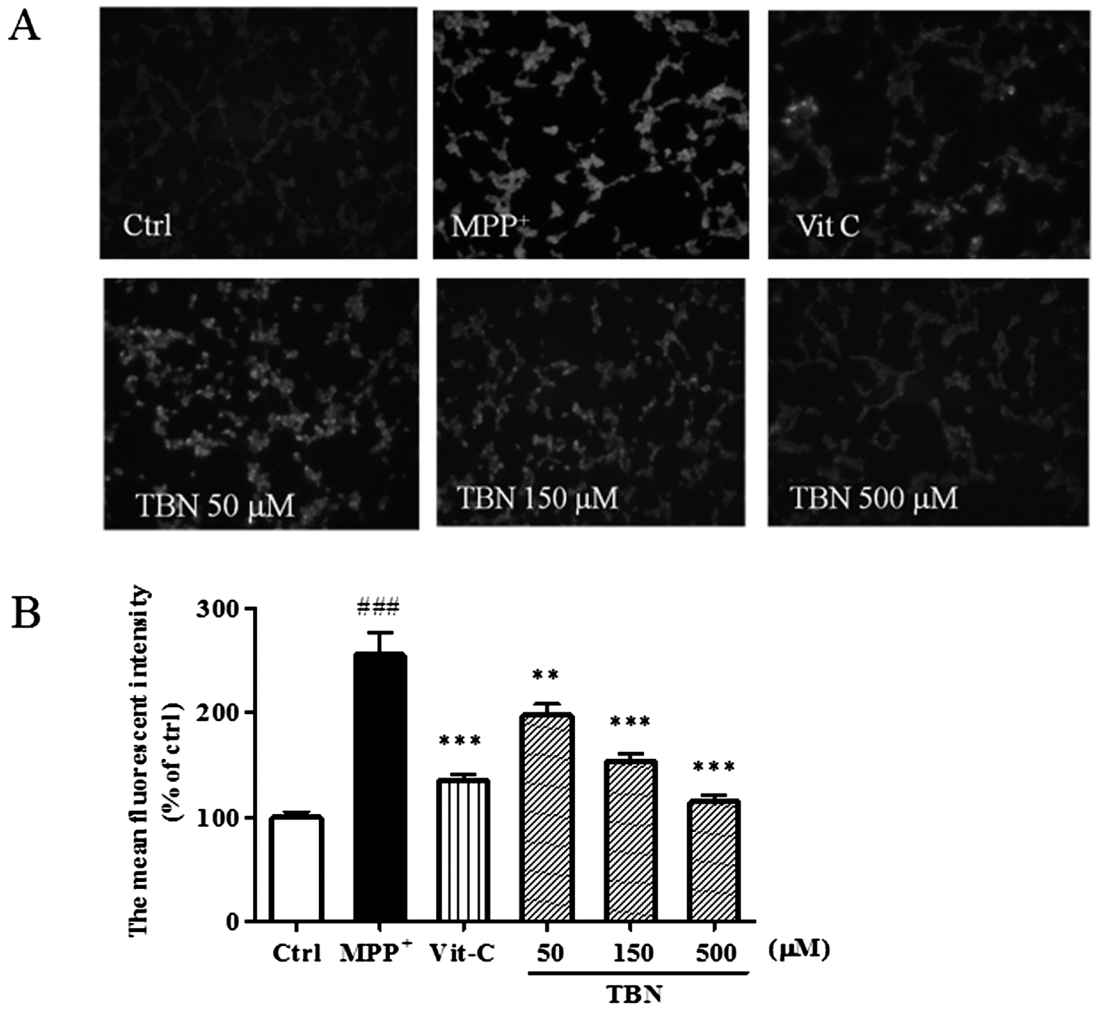

Fig. 4. TBN Lowered Intracellular ROS Level in SH-SY5Y Cells Induced by MPP^+

(A) Representative pictures of intracellular ROS determined by DCFH-DA staining. (B) Quantification of fluorescent intensity of DCFH-DA in the different treatment groups. Data were from three independently experiments. $^{###}p<0.01$ *versus* Ctrl group; $^*p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ *versus* MPP^+ group.

number of SH-SY5Y cells and changed the cell morphology. For example, some cells became rounded, and others became detached from adjacent cells. TBN significantly blocked the loss of cells and reversed the morphological alteration induced by MPP^+. As shown in Figs. 2B and C, MPP^+ significantly decreased the viability of SH-SY5Y cells and increased the round-shaped cells. TBN protected cells from MPP^+-induced cell death and morphological change in a dose dependent manner and achieved maximal efficacy at 500 μM.

TBN Prevents MPP^+-Induced Damage of Primary Midbrain Dopaminergic Neurons Exposure to MPP^+ (2 μM) for 48 h significantly decreased the number of TH-positive dopaminergic neurons, resulting in a *ca.* 60% reduction compared to the control group, accompanied by neurites shortening or even disappearing. However, 2 h pretreatment with TBN or positive control drug selegiline (a selective MAO-B inhibitor) significantly and concentration-dependently prevented the MPP^+-induced neuronal damage. It also maintained the neurite length of TH-positive cells (Figs. 3A, B).

Effect of TBN on MPP^+-Induced ROS Production To confirm that ROS-scavenging is involved in the protection of TBN against MPP^+ toxicity, SH-SY5Y cells were treated with MPP^+ for 24 h before the intracellular ROS levels were determined using the fluorescence probe DCFH-DA. ROS level increased by 2.5-fold compared to that of the control group after exposure of MPP^+. Remarkably, pretreatment with TBN (50–500 μM) for 1 h significantly decreased the ROS level in a concentration-dependent manner (Fig. 4).

Neuroprotection of TBN on MPTP-Induced Toxicity in Dopaminergic Neurons of Zebrafish The similarity of the development of DA neurons in zebrafish with other vertebrates makes it a good model to study the disorders of the DA system.[30] To further investigate the neuroprotective effect of TBN, we manifested the DA neurons in zebrafish by whole-mount immunostaining using an antibody against tyrosin-hydroxylase (TH). As shown in Fig. 5, exposure to MPTP for 48 h resulted in approximately 70% loss in TH-positive neurons in the diencephalic area of zebrafish embryos. TBN (added simultaneously with MPTP into the fish embryo medium) significantly prevented the neuron loss induced by MPTP. Both control compounds, *i.e.*, the parent compound TMP (500 μM) and selegiline, showed protection against the MPTP-induced DA neuron loss.

Effect of TBN on TH-Positive Cells and Levels of Dopamine and Its Metabolites in MPTP Treated Mice We further investigated the *in vivo* neurorestorative effects of TBN in

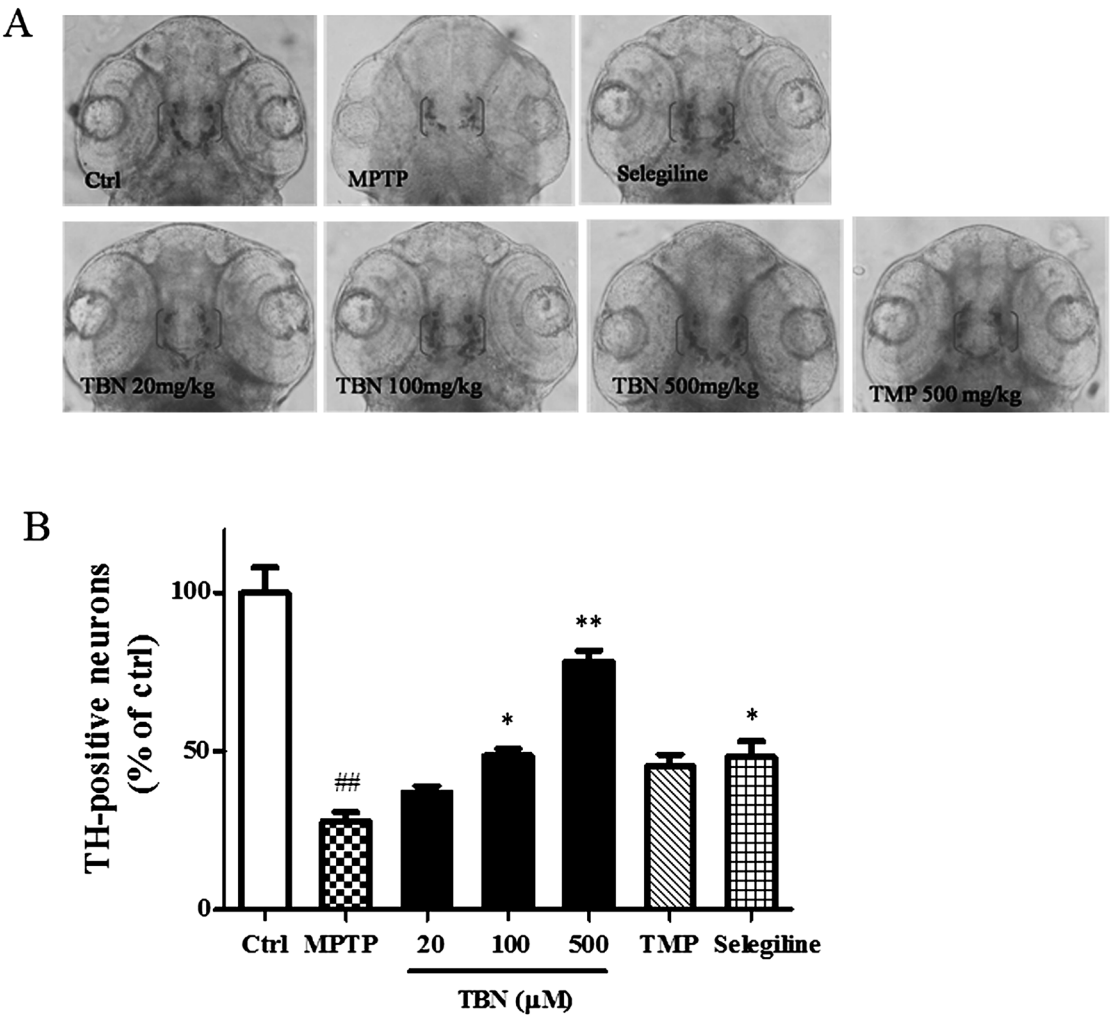

Fig. 5. TBN Attenuated the Loss of Dopaminergic Neurons in MPTP-Lesioned Zebrafish

TBN and MPTP were added simultaneously into the fish embryo medium and the treatment period was 48 h. (A) Representative photographic images of dopaminergic (TH-positive) neurons. (B) The number of TH-positive neurons. Data were from 3 independent experiments, and were expressed as percentage of the vehicle control group. $^{\#\#}p<0.01$ *versus* Ctrl group; $^*p<0.05$ and $^{**}p<0.01$ *versus* MPP^+ group.

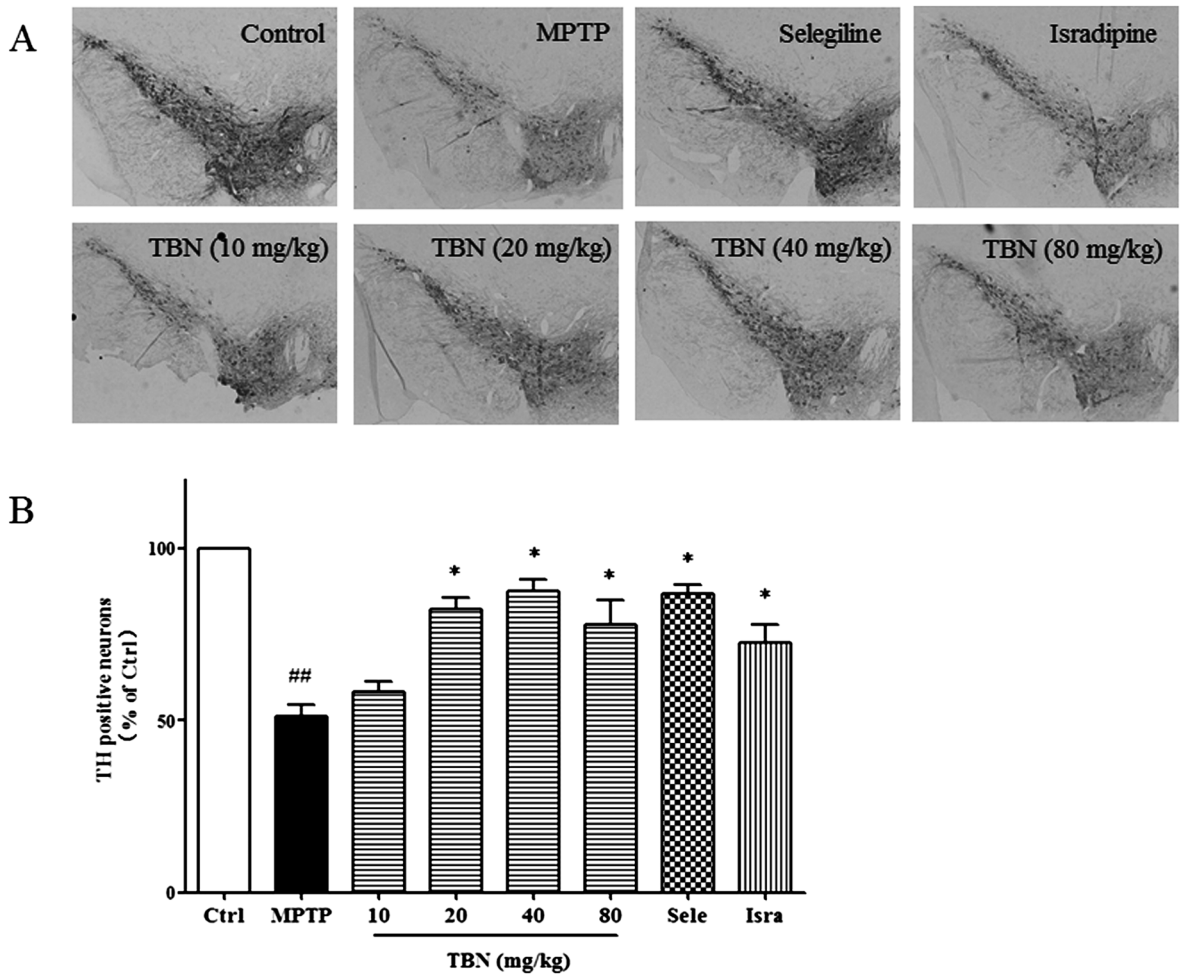

Fig. 6. TBN Attenuated the Loss of Dopaminergic Neurons in MPTP-Lesioned Mice

TBN was administrated orally twice daily for 14 d at 3 d post-MPTP lesion in mice. (A) Representative photographic images of dopaminergic (TH-positive) neurons. (B) The mean number of TH-positive neurons. Sele, selegiline; Isra, isradipine. Data were expressed as percentage of the Ctrl group (mean±S.D.; $n=6$ for all treatment groups). $^{\#\#}p<0.01$ *versus* Ctrl group; $^{*}p<0.05$ *versus* MPP^{+} group.

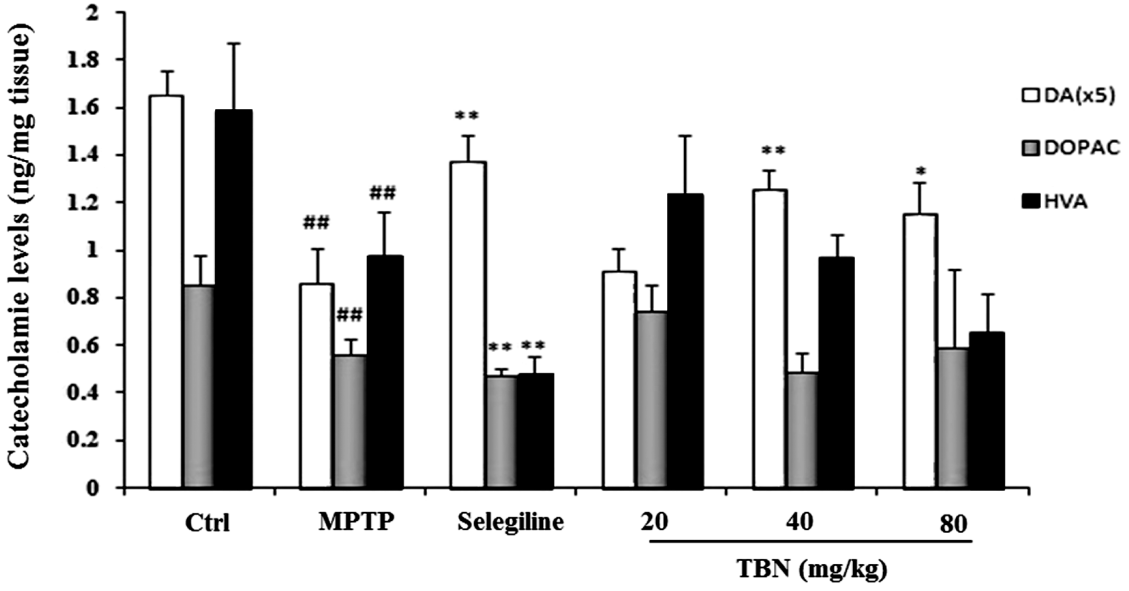

Fig. 7. Effect of TBN on Striatal DA, DOPAC, and HVA in MPTP-Treated Mice

TBN was administrated orally twice daily for 14 d at 3 d post-MPTP lesion in mice. Results were expressed as percentage of the Ctrl group (mean±S.D., $n=6$ for all treatment groups). 'DA (×5)' means the absolute levels of DA is 5 times of the values indicated. $^{\#\#}p<0.01$ *versus* Ctrl group; $^{*}p<0.05$ and $^{**}p<0.01$ *versus* MPP^{+}-treated group.

a MPTP-treated mouse model of PD. In this model, MPTP can induce SNpc neuron loss. As shown in Fig. 6A, MPTP treatment remarkably reduced the number of TH-positive neurons in the SNpc, visualized by immunostaining of TH expression. MPTP produced approximately 40% of neuron loss in SNpc (Fig. 6B). In contrast, TBN (20, 40 and 80 mg/kg administrated orally twice daily at 3 d post-MPTP lesion for 14 d) significantly rescued the MPTP-induced loss of TH-positive neurons. Isradipine is a classic L-type Ca^{2+} channel inhibitor. Recent researches in animal models suggest that isradipine may have potential uses for treating PD, resulting from its blockage of $Ca_v1.3$ Ca^{2+} channels.[39] Isradipine applied as a control here also significantly rescued dopaminergic neurons loss. Importantly, TBN at 40 mg/kg was as effective as the clinically used anti-PD drug selegiline. At 80 mg/kg, the efficacy of TBN decreased probably due to increased toxicity.

In order to find out the relationship between the number of TH-positive cells and the contents of striatal dopamine as well as its metabolites, we determined the levels of striatal dopamine and its metabolites using electrochemical HPLC. MPTP administration caused a significant reduction in the levels of dopamine and its metabolite HVA and DOPAC in the striatum, compared with that of the control group (Fig. 7). The reductions of dopamine level were attenuated when the animals were treated with selegiline or with 40 and 80 mg/kg TBN. However, there was no significant impact on MPTP-induced reduction of HVA and DOPAC level after TBN treatment.

TBN Elevates SOD Activity and GSH Level in the Striatum of the MPTP Treated Mice Based on the significant neuroprotective and antioxidative effects of TBN observed in our *in vitro* MPP^+ models (Figs. 2–4), we examined its antioxidative capacity in the MPTP-treated mice. The SOD activity and GSH content decreased significantly in the substantial nigra of mice following MPTP treatment (Table 1). TBN treatment dose-dependently elevated the SOD activity and GSH level. However, selegiline at 10 mg/kg did not show these properties in the same model.

Therapeutic Effect of TBN on Unilateral 6-OHDA-Lesioned Model of PD in Rats Six-OHDA is a neurotoxin that selectively destroys DA neurons. The advantage of this model is that it is easier to assess the side-biased motor impairments by utilizing drug-induced rotation tests.[40] In the present study, we validated whether TBN could also preserve DA neurons against 6-OHDA-induced degeneration. As shown in Fig. 8, at 3 weeks post the unilateral SNpc and VTA 6-OHDA injections, the number of DA neurons in both the SNpc and the VTA significantly decreased. The cell loss in the SNpc on the lesioned side reached over 90% (from 167±29 to 12±7). There was no marked difference in TH cell numbers in the contralateral SNpc between the lesioned and control mice. Hematoxylin counterstaining showed that 6-OHDA injection resulted in cell inflammation, especially in microglial cells. The inflammation infiltrated into the lesioned areas, and the number of microglial cells clearly increased (indicated by arrow heads) in the right column of Fig. 8A. At 7 d post-lesion, TBN (45 mg/kg) administrated orally twice daily for 14 d significantly rescued the loss of DA neurons induced by 6-OHDA (41±15). The positive control agent selegiline also significantly reduced the loss of DA neurons. L-Dopa had no effect. TBN at all doses tested inhibited the 6-OHDA-induced microglia infiltration.

All of the rats with 6-OHDA-induced PD showed significantly higher rotational numbers at 7 d after stereotaxic surgery compared to those of normal or sham-operated groups (data not shown). As shown in Fig. 9, at the end of the drug treatment, apomorphine-induced rotational behavior was tested again. TBN treatment after 6-OHDA injection significantly improved the rotational behavior in a dose-dependent manner compared to those without TBN treatment. The positive control agent L-dopa also significantly improved the rotational behavior.

DISCUSSION

In the present study, our results showed that the significantly effective concentrations of TBN were among 50–500 μM *in vitro*, and the effective doses of TBN by oral administration were among 15–80 mg/kg in rodent model. Therefore, what concentration can TBN reach in the brain following 80 mg/kg of TBN administrated orally? Previously, we have demonstrated that TBN could easily cross the blood brain barrier. TBN concentration in the rat brain tissues reached 147.3 μg/g (approximately 650 μM, assuming the volume of 1 g brain tissue is 1 mL) at 5 min following 80 mg/kg of TBN administration, and the brain concentration at over 28 μg/g (approximately 125 μM) lasted 6 h.[27]

The free radicals most destructive to tissues are $\cdot OH$, $\cdot O_2^-$ and $ONOO^-$. Unfortunately, until now, no single agent could effectively neutralize all three at the same time. TMP has been reported to scavenge $\cdot OH$, $\cdot O_2^-$ and $\cdot NO$,[16] but its efficacy against $ONOO^-$ has not been investigated. Nitrones such as PBN are effective at scavenging $\cdot NO$, $\cdot OH$, and $\cdot O_2^-$.[41,42] PBN's ability to scavenge $ONOO^-$ directly has not been investigated.[43] TBN is designed to have properties of both TMP and the nitrone moieties. Previously we demonstrated that the natural TMP was highly effective against $\cdot OH$, but had

Table 1. TBN Elevated SOD Activity and GSH Level in the Substantial Nigra of MPTP Treated Mice

Group	Dose (mg/kg)	SOD (U/mg protein)	GSH (ng/mg protein)
Control	—	73.2±7.86	39.0±2.02
MPTP	—	37.7±9.02#	22.0±0.61#
MPTP+TBN	20	49.5±7.51	33.5±4.10
MPTP+TBN	40	57.6±4.77*	37.1±6.60*
MPTP+TBN	80	54.8±9.50*	36.2±5.20*
MPTP+Selegiline	10	44.8±7.45	32.9±5.08

TBN was administrated orally twice daily for 14 d at 3 d post-MPTP lesion in mice. Results were expressed as mean±S.D. (n=6 for all groups). #$p<0.01$ *versus* to Ctrl group; *$p<0.05$ *versus* MPP^+ group.

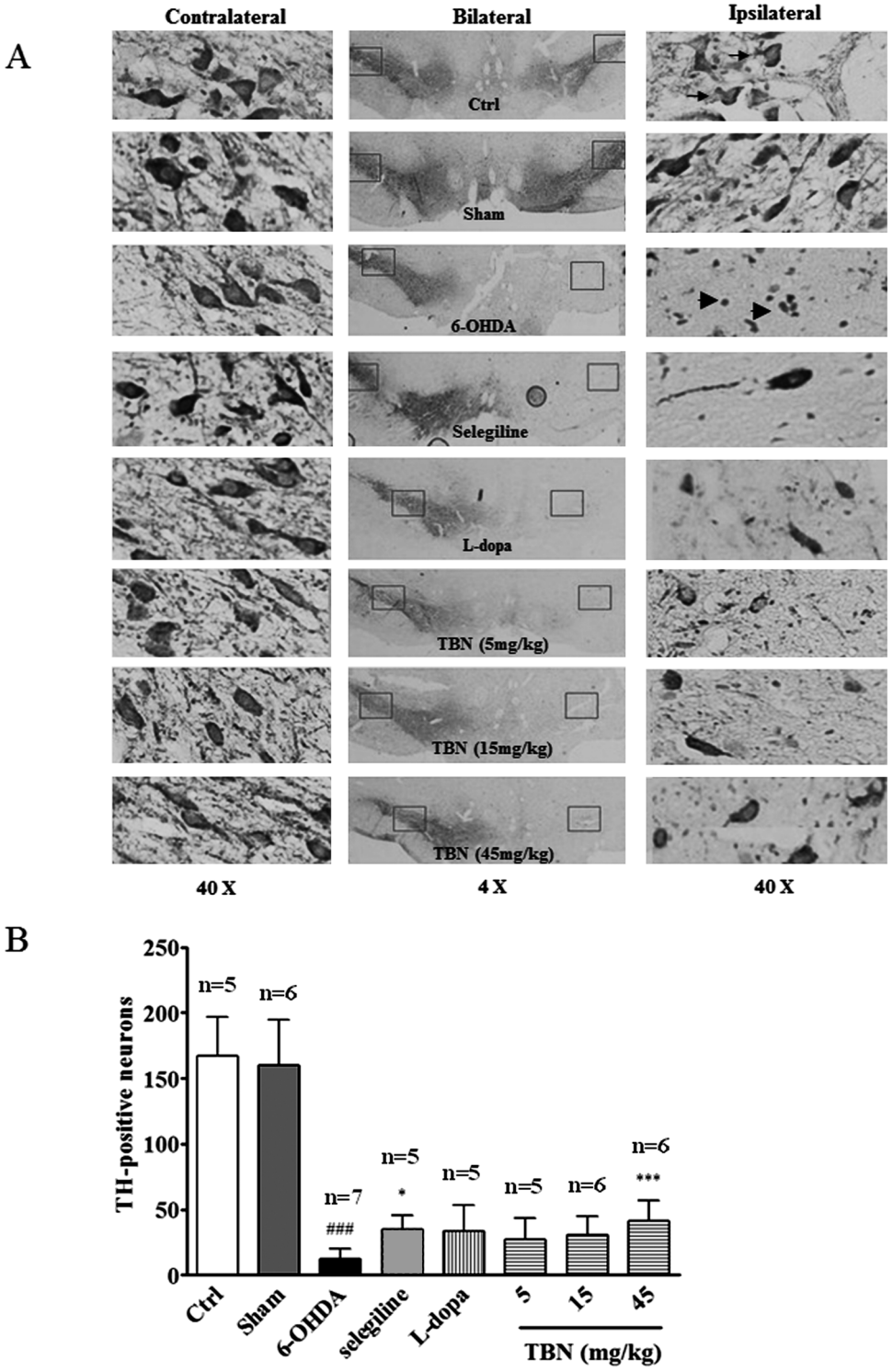

Fig. 8. TBN Increased the Number of Surviving SNpc DA Neurons in Animals Treated with 6-OHDA

TBN was administrated orally twice daily for 14 d at 7 d post-6-OHDA lesion in rats. (A) Representative microphotographs demonstrating TH-positive DA neurons in the SNpc of rat brain. Middle column showed the bilateral of brain slices (4×). Left and right columns represented the higher magnification (10×) of the red square-indicated areas in the contralateral and the ipsilateral of 6-OHDA-leisioned brain, respectively. Arrows indicated the TH-positive DA neurons, and arrow heads indicated the astrocytes. (B) The mean number of TH-positive neurons per section in the ipsilateral of 6-OHDA-leisioned brain. Data were expressed as mean±S.D. $^{###}p<0.001$ *versus* Ctrl group; $^{*}p<0.05$ and $^{***}p<0.001$ *versus* 6-OHDA-treated group.

minimal activity against $\cdot O_2^-$ and $ONOO^-$. PBN had modest activity against $\cdot OH$ and $ONOO^-$ but was inactive against $\cdot O_2^-$. In sharp contrast to both TMP and PBN, the new TBN was highly effective against all three.[27] It is widely reported that antioxidants prevent MPTP/MPP^+-induced neurotoxicity by scavenging free radicals. In the present study, exposure of SH-SY5Y cells to MPP^+ resulted in a 2.5-fold increase in intracellular ROS level. Remarkably, pretreatment with TBN (50–500 μM) for 1 h significantly decreased the ROS level in a concentration-dependent manner. TBN at a concentration of 500 μM was as effective as the positive control, Vit-C at 100 μM, completely reversing the elevated ROS level to normal

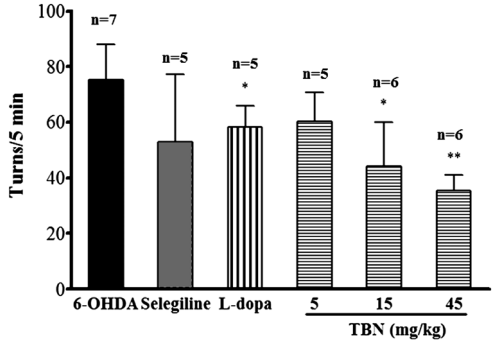

Fig. 9. TBN Improved the Apomorphine-Induced Rotational Behavior in 6-OHDA-Induced Rat PD Model

TBN was administrated orally twice daily for 14 d at 7 d post-6-OHDA lesion in rats. Data were expressed as average turns per min of each group (mean±S.D.). $*p<0.05$ and $**p<0.01$ *versus* 6-OHDA-treated group.

(Fig. 4). TBN's strong and unique antioxidative effects will undoubtedly contribute to its neuroprotective and therapeutic efficacy against PD.

The generation of ROS is a part of normal cellular metabolism, and cells have a variety of defense mechanisms to scavenge free radicals.[44] These include the conversion of $\cdot O_2^-$ to H_2O_2 catalysed by the enzyme SOD, as well as the reaction of H_2O_2 with reduced glutathione (GSH) to produce water controlled by glutathione peroxidase. Post-mortem studies in the PD brain indicate that those affected by PD have a metabolic defect in the DA neurons.[45,46] One such defect could result in a decreased production of scavengers. The diminished levels of GSH and other defensive antioxidants reported in PD may exacerbate this problem.[47] In our current work, SOD activity and GSH content were significantly decreased in the substantial nigra of mice following MPTP treatment. MPTP treatment destroyed the antioxidative defense system of PD mice, thus rendering the DA neurons more susceptible to oxidative injury. TBN treatment dose-dependently elevated SOD activity and GSH levels in MPTP-injected mice (Table 1). Our result indicates that TBN's effect in increasing cellular antioxidative defense may also contribute to its neuroprotective and therapeutic efficacy against PD.

Increasing evidence suggests that neuroinflammatory processes contribute to the cascade leading to the progressive neuronal damage in PD.[8] Upregulation of pro-inflammatory gene expression and increase of activated microglia were observed in 6-OHDA-induced PD models.[9,10] In the current study, we found that microglial cells activated and infiltrated into the substantial nigra of the unilateral 6-OHDA-lesioned model of PD. TBN administration obviously increased the number of microglial cells while increasing the survival of TH-positive DA neurons (Fig. 8). Previous studies demonstrated that TMP exerted neuroprotection by reducing inflammatory cell activation and proinflammatory mediator production in the ischemic stroke model.[18,19] TBN may retain or even improve upon these anti-inflammatory properties, which are beneficial to neuroprotection; however, this remains to be further demonstrated experimentally.

Excitotoxicity and calcium overload have been implicated as pathogenic mechanisms in PD. Excessive *N*-methyl-D-aspartate (NMDA) receptor activation by glutamate could increase intracellular calcium levels that then activate cell death pathways.[48] Epidemiological studies have suggested that hypertension patients treated with L-type calcium blocker dihydropyridines have a lower incidence of PD.[49] The dihydropyridine isradipine is currently being investigated as a potential neuroprotective agent in clinical trials in PD patients.[50] In the present study, isradipine also significantly rescued dopaminergic neurons loss induced by MPTP in mice. Studies have shown that TMP, TBN's parent compound, is an L-type Ca^{2+} channel blocker.[15] Moreover, we previously demonstrated that TBN significantly reduces Ca^{2+} entry into cells induced by KCl, possibly by blocking the L-type Ca^{2+} channels.[27] Therefore, the neuroprotective effect of TBN in the current study may also be mediated by the blockage of calcium influx.

Because of the complex nature of PD, it may be advantageous or even essential that therapeutics for PD treatment acts through multiple mechanisms of action, *i.e.*, by targeting multiple pathological pathways at the same time. In our current and previous works, TBN acts through at least three mechanisms. First, TBN decreases ROS production by blocking Ca^{2+} overload in cells, probably through antagonizing the L-type calcium channels. Second, TBN directly scavenges all three of the most damaging radicals to cells, *i.e.*, $\cdot OH$, $\cdot O_2^-$ and $ONOO^-$, due to its unique chemical structure. Thirdly, TBN may also act by reducing neuroinflammation.

Although the precise molecular events involved in the neuroprotective and therapeutic effects of TBN need to be further elucidated experimentally, it is clear that TBN is multifunctional. TBN's mechanisms of action are unique, and no other agent is reported to have such multiple capabilities thus far.

Acknowledgments This work was supported in part by Grants from the China Natural Science Fund (U1032030973618 to YW, and NSFC 81303251 to ZZ), the China Innovative Drug Project (2012ZX09103-055 to YW) and the 211 project of Jinan University, as well as the Science and Technology Development Fund of Macau SAR (Ref. no.: 014/2011 and 078/2011/A3) and the Research Committee, University of Macau. We thank Ms. Linda Wang of School of Medicine at University of California, San Francisco for proofreading of the manuscript.

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