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Neuroprotective effects of TEMPOL in central and peripheral nervous system models of Parkinson's disease

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Abstract

TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl) is a stable nitroxyl antioxidant. Previous studies have suggested that TEMPOL is protective in acute disorders thought to involve reactive oxygen species (ROS), such as ischemic stroke and cardiac reperfusion injury. Oxidized TEMPOL can be recycled to its redox-active reducing form by co-administration with polynitroxylated albumin, making it a candidate as a pharmacological ''reservoir'' for reducing potential of use in chronic disorders involving ROS. The present studies examine the efficacy of TEMPOL in cell culture and animal models of the central and peripheral dysfunction associated with Parkinson's disease, a disorder in the pathogenesis of which ROS generated from dopamine have been implicated. Antioxidants have been proposed as both preventive and symptomatic therapy for Parkinson's disease. TEMPOL protects MN9D dopaminergic mesencephalic cells in culture from 6-hydroxydopamine (6-OHDA)-induced apoptosis. Translocation of the p65 component of NF-κB to the nucleus accompanies protection by TEMPOL. In vivo, intraperitoneal TEMPOL protects mice from intrastriatal 6-OHDA-induced cell and dopamine metabolite loss in the striatum. TEMPOL also protects mice against the 6-OHDA-induced rotational behavior elicited by intrastriatal administration of D-amphetamine. In addition, TEMPOL protects mice from the ptosis, activity level decrement, and mortality induced by intraperitoneal administration of 6-OHDA, a model of autonomic dysfunction in Parkinson's disease. Adjunctive use of polynitroxylated albumin enhances the in vitro and in vivo effects of TEMPOL.

Keywords: Parkinson's disease; Reactive oxygen species; Antioxidants; Neuroprotection; Dopamine; Autonomic nervous system

1. Introduction

Parkinson's disease is a neurodegenerative disorder that primarily affects dopaminergic neurons in the central nervous system. Peripheral neurons, particularly those in the autonomic nervous system, can be affected as well [1,2]. The tendency of dopamine to oxidize forming reactive oxygen species (ROS) has been proposed to be a component of the pathogenesis of Parkinson's disease. Under normal circumstances, endogenous antioxidant mechanisms serve to detoxify those ROS formed along with neuromelanin in dopaminergic neurons. When these mechanisms fail, it has been hypothesized and demonstrated in cell culture and animal model systems, these dopaminergic neurons are injured and die, often by apoptosis [3–8].

Both abortive and preventive approaches to Parkinson's disease have included the use of antioxidant drugs. Oxygen radical scavengers and reducing agents, like Vitamins C and E, have not, however, shown unequivocal promise in this regard ([9,10]; reviewed in [11]). It has been suggested that this is because, although they rapidly reduce ROS, themselves becoming oxidized, their redox potential is such that under physiological conditions, they are not readily re-reduced to their active scavenging form [12–14]. That is,

Abbreviations: TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl; PNA, polynitroxylated albumin; DOPAC, dihydroxyphenylacetic acid; HVA, homovanilic acid; TH, tyrosine hydroxylase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; i.p., intraperitoneal; i.v., intravenous

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they are effectively depleted after one redox cycle. Both non-scavenging, indirect ROS detoxifiers and recycling antioxidants have been suggested as alternatives to these direct ROS scavengers [14,15].

TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl) is an antioxidant that works as a superoxide dismutase mimic [16], directly reacts with both carbon-centered and peroxy radicals [17], and prevents the reduction of hydrogen peroxide to the hydroxyl radical [18]. Furthermore, this nitroxide can oxidize reduced transition metals that would otherwise serve to catalyze the formation of the hydroxyl radical via the Fenton reaction [19]. We have previously shown that TEMPOL decreases the mortality rate of mice treated intraperitoneally (i.p.) with the ROS-generating dopamine analogue, 6-hydroxydopamine (6-OHDA) [14,20], and that prolonging the half-life and decreasing the toxicity of TEMPOL with polynitroxylated albumin (PNA) enhances this protective effect [14]. The present investigation examines the neuroprotective effects of TEMPOL in in vitro and in vivo models of the central nervous system damage and dysfunction associated with Parkinson's disease. It further demonstrates protection of autonomic nervous system function by TEMPOL in an in vivo model of peripheral nervous system dysfunction associated with Parkinson's disease. PNA enhances these in vivo effects of TEMPOL.

2. Materials and methods

2.1. Chemicals

4-Hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL), dihydroxyphenylacetic acid (DOPAC), homovanilic acid (HVA), dopamine, 6-hydroxydopamine (6-OHDA) and D-amphetamine sulphate were purchased from Sigma Chemical Corporation (St. Louis, MO, USA) at the highest level of purity available. PNA was obtained from SynZyme Technologies, LLC (Irvine, CA, USA) and prepared for use as has previously been described [13,14].

2.2. Cells

The dopaminergic MN9D cell line is a fusion of rostral mesencephalic neurons from embryonic C57BL/6J (embryonic day 14) mice with N18TG2 neuroblastoma cells. These cells, which express both tyrosine hydroxylase (TH) and aromatic acid decarboxylase and produce measurable levels of dopamine, were a kind gift of Drs. A. Heller and L.A. Won (University of Chicago).

2.3. Cell culture

MN9D cells were maintained in Dulbecco's minimal essential medium (MEM; Sigma) supplemented with 10% FBS (Hyclone) at a density of 1.2×10^6 cells on 100 mm primaria culture dishes (Becton Dickinson, Lincoln Park, NJ, USA). Prior to each experiment, MN9D cells were differentiated by maintenance in DMEM supplemented with 10% FBS and 1 mM *n*-butyrate (Sigma) for 5–7 days.

2.4. In vitro treatment with 6-OHDA; determination of cell survival after treatment

The survival of MN9D cells after treatment with 6-OHDA was assessed using the tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS; Promega, Madison, WI, USA), and the following procedure: MN9D cells were cultured in 96-well plates (1.2×10^4 cells per well) and treated with 1 mM *n*-butyrate for 5–7 days to induce differentiation. After treatment with 6-OHDA for 20 min, the cells were washed once with fresh medium and incubated for another 24 h. The MTS assay was then performed. In brief, cells were placed in fresh medium (100 μl) and 20 μl MTS reagent was added to each well. After incubation for 1 h at 37 °C, the medium was seen to develop an orange color. The $OD_{570\ nm}$ was determined for each well using a microplate reader (Spectra MAX 340, Molecular Devices). Values from each treatment paradigm were compared with vehicle-treated control.

2.5. Immunocytochemistry; examination of cells for translocation of NF-κB p65 component

MN9D cells were incubated with TEMPOL or vehicle for 1 h, washed three times, and then incubated with 6-OHDA or vehicle for the duration of the experiment. The cells were then washed three times with 1× PBS, fixed in 95% ethanol for 20 min, and washed three more times with 1× PBS. The cells were then permeabilized by incubation in 2% Triton X-100 for 30 min followed by three 1× PBS washes. After blocking of non-specific staining (0.5% bovine serum albumin + 2% horse serum in PBS; 10 min), the cells were incubated with anti-p65 antibody for 2 h at 37 °C and then with a 1:100 dilution of goat anti-rabbit antibody-Cy™3 immunoconjugate followed by three additional washes (10 min each) with 1× PBS. The membranes were removed from the wells and placed onto glass microscope slides to examine the translocation of NF-κB p65 component from cytoplasm to nucleus using a Zeiss light microscope equipped for epifluorescent illumination. Non-specific immunostaining (negative control) was assessed by subjecting sister cultures to this same procedure minus treatment with the primary antibody.

Western blotting for quantification of IκBα and p-IκBα: the NF-κB complex component, IκBα, and its phosphorylated counterpart, p-IκBα, were detected and measured in MN9D cells by Western blotting and densitometry as we have previously described [21].

2.6. Animals

All procedures were carried out in accordance with the National Institutes of Health's "Guide for Care and Use of Laboratory Animals" and were approved by the Institutional Animal Care and Use Committees of the Children's Hospital of Pittsburgh and the University of Pittsburgh School of Medicine. Adult A/J mice (25 ± 4 g) were housed in groups of four in air-conditioned quarters at a temperature of 22 °C and maintained on a 12-h light/dark cycle. They had free access to food and water at all times.

2.7. Treatment of mice with 6-OHDA and/or TEMPOL

For intrastriatal treatment with 6-OHDA, animals were injected i.p. with TEMPOL (200 mg/kg) or an equivalent volume of vehicle 60 min prior to surgical incision. The anesthetic agent, Equithesin, was administered i.p. 10 min prior to surgical incision. The animals were placed in a stereotaxic apparatus, and a point directly dorsal to the left striatum (from bregma: 1.0 mm lateral, and 0.8 mm anterior, 2.5 mm ventral [22]) was marked. After drilling a hole, a 26-gauge steel cannula with polypropylene microtubing attached to a 10-μl Hamilton glass microsyringe was slowly lowered into the brain to a depth of 2.8 mm ventral to the surface of the brain, and vehicle and or 6-OHDA was delivered at a rate of 0.1 μl/min with a digital mini-pump.

I.p. treatment with 6-OHDA was performed as we have previously described [14,20].

2.8. Measurements of TEMPOL in brain homogenates

EPR measurements of TEMPOL were performed on a JEOL JES-RE1X spectrometer at 25 °C after tail vein (i.v.) injection of TEMPOL into mice. Redox-activity and total recyclable TEMPOL in brain homogenates in their native state and treated with potassium ferricyanide (to ensure that all of the TEMPOL was in the reduced and therefore ESR-detectable state), respectively. Whole mouse brain was homogenized in 500 μl PBS. EPR measurements were performed in gas-permeable Teflon tubing (0.8 mm internal diameter, 0.013 mm thickness) obtained from Alpha Wire Corp. (Elizabeth, NJ, USA) on a JEOL JES-RE1X spectrometer at 25 °C. The Teflon tube (approximately 8 cm in length) was filled with 75 μl of brain homogenate, folded in half, and placed into an open EPR quartz tube (inner diameter of 3.0 mm). EPR spectra were recorded at 335.1 mT, center field; 10 mW, power; 0.05 mT, field modulation; 5 mT, sweep width; 100 and 6300, receiver gain; 0.1 s, time constant. Spectra were collected using EPRware software (Scientific Software Services, Bloomington, IL, USA).

2.9. Tissue preparation for assay of dopamine, DOPAC, and HVA

Animals were killed by rapid decapitation 1 week after treatment with 6-OHDA and/or TEMPOL with or without PNA, and their brains were rapidly removed. The brains were dissected over an ice-cold platform. The striatum was isolated from each and sonicated in 1000 μl of ice-cold buffer. The resulting samples were centrifuged at 4 °C at $17{,}000 \times g$ for 25 min and the supernatant was filtered through a 0.22 μm filter and stored at −70 °C until HPLC analysis for dopamine, DOPAC, and HVA was performed.

2.10. HPLC analysis of dopamine, DOPAC, and HVA

Analysis of the tissue content of DOPAC, dopamine, and HVA was performed by HPLC as was previously described [23]. An ESA HPLC system was used.

2.11. Tissue preparation for immunohistology

Two weeks after treatment with 6-OHDA and/or TEMPOL, mice were killed by CO_2 narcosis and perfused with 4% paraformaldehyde dissolved in saline. Their brains were removed and kept in 4% paraformaldehyde for 2 h, then incubated in 30% sucrose for 48 h at 4 °C. Brains were sectioned on a freezing microtome at 40 μm thickness. Sections were then stored in anti-freezing buffer at −20 °C until staining and examination.

2.12. Immunohistochemistry

Every sixth section through the substantia nigra was processed free-floating for TH immunostaining. Tissue sections were incubated first in 1% H_2O_2 to quench the endogenous peroxidases in the tissue and then in 2% horse serum in PBS for 1 h to block the non-specific binding of immunoreagents. After subsequent maintenance in 1% Triton X-100/PBS (Sigma) for 20 min, the sections were incubated at 4 °C for 48 h with monoclonal anti-TH antiserum (Chemicon Inc., Temecula, CA, USA) diluted 1:2000 in 2% horse serum in PBS. They were then washed three times with PBS, incubated with biotinylated horse anti-mouse IgG (1:1000; Vector Laboratories, Burlingame, CA, USA) for 1 h, washed three times, and incubated with avidin–biotin–peroxidase solution (ABC kit; Vector Laboratories) for 1 h. Immunolabeled sections were then washed three times in PBS and stained using 3,3-diaminobenzidine (DAB kit; Vector Laboratories) to detect the immunocomplexes. Stained cells were then counted manually by microscopy.

2.13. Rotational behavior tests

Mice were tested for rotational behavior induced by D-amphetamine (2.5 mg/kg, i.p.) 6 weeks after treatment with 6-OHDA. Rotations were counted over 5 min intervals for 35–40 min [24].

2.14. Statistical analysis

In the case of comparisons of normally distributed variables between two groups, statistical significance was determined using Student's *t*-test. In the case of comparisons of normally distributed variables among multiple groups, statistical significance was determined using one-way analysis of variance (ANOVA), followed by the Fisher protected least significant differences (PLSD) post-hoc test. In the case of comparisons of non-parametric variables, statistical significance was determined using the Mann–Whitney *U*-test. In all cases, p-values <0.05 were deemed to be statistically significant.

3. Results

3.1. TEMPOL in in vitro and in vivo models of the central nervous system manifestations of Parkinson's disease

3.1.1. Effects of TEMPOL on the viability of MN9D dopaminergic neuronal cells treated with 6-OHDA

Treatment of MN9D cells with 6-OHDA (100 μM) for 20 min results in death of 30% of the cells 24 h later. Pretreatment with TEMPOL (0.15 μM) 1 h before addition of 6-OHDA results in a reduction in 6-OHDA-induced cell death at 24 h to 5% of the cells (Fig. 1A and B). In fact, increased cell survival is seen in 6-OHDA-treated cells even if TEMPOL is added 0.5 h after 6-OHDA addition to the cultures (Fig. 1B). TEMPOL significantly reduces cell death, and PNA significantly enhances this effect at all concentrations of 6-OHDA tested (Fig. 1C). PNA alone is without effect in this model (data not shown).

3.1.2. Effects of TEMPOL on activation and nuclear translocation of NF-κB in MN9D cells

Immunofluorescent visualization of the p65 subunit of NF-κB indicates that nuclear translocation of this protein is evident in MN9D cells by 60 min after treatment with TEMPOL (Fig. 2A). Treatment with 6-OHDA alone does not induce nuclear translocation of p65, nor does it prevent TEMPOL-mediated translocation. These results mirror those we have previously published for PC12 cells [14]. They were confirmed biochemically by demonstration that IκBα is phosphorylated to p-IκBα in MN9D cells by 60 min after treatment with TEMPOL or TEMPOL-6-OHDA, but not after treatment with 6-OHDA alone (Fig. 2B). Phosphorylation of IκBα is not seen at 10 or 180 min after any of these treatment regimens (data not shown).

3.1.3. Entry of peripherally administered TEMPOL into the central nervous system

Peripherally administered TEMPOL readily enters and is detected in the brain. Fig. 3 shows the dose–brain

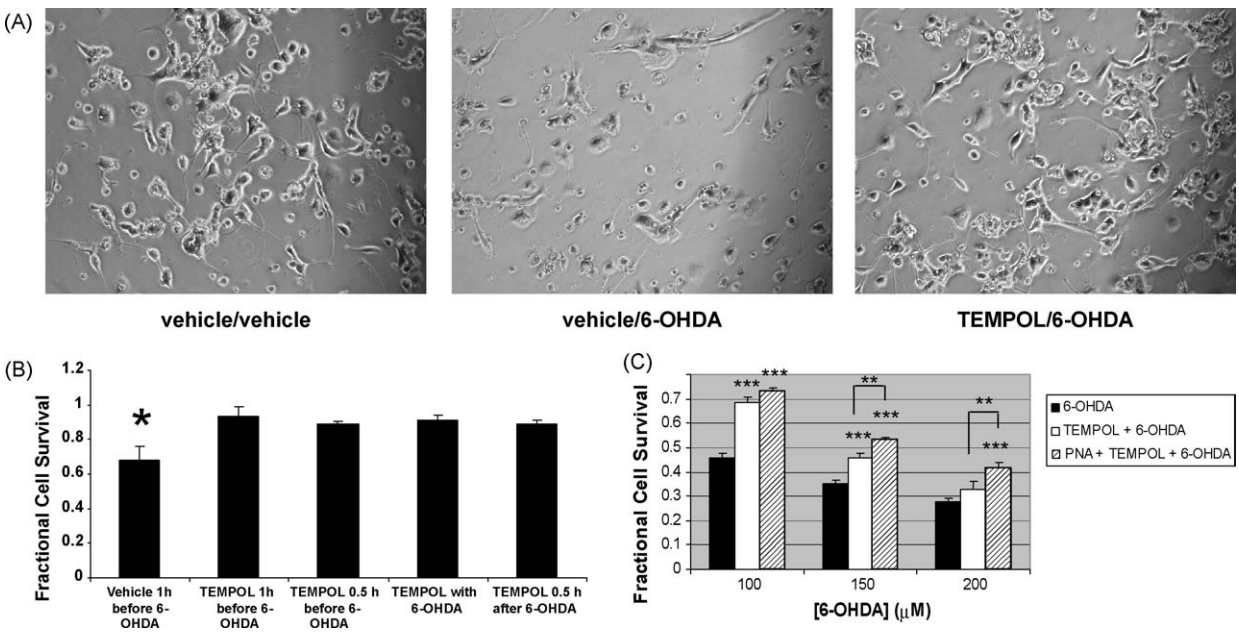

Fig. 1. Effects of TEMPOL on 6-OHDA-induced MN9D dopaminergic neuronal cell death. (A) Light micrographs of MN9D cells treated with vehicle, vehicle followed 1 h later by 6-OHDA (100 μM), or TEMPOL (0.15 μM) followed by 6-OHDA (100 μM). Magnification, 400×. (B) Time-course of enhancement by TEMPOL of 6-OHDA-treated MN9D cell survival. MN9D cells were treated with TEMPOL (0.15 μM) at various time points relative to the addition of 6-OHDA (100 μM) to the culture medium. 6-OHDA was left in the medium for 20 min, after which time fresh medium containing TEMPOL was added. TEMPOL was maintained in the medium for an additional 24 h, at which time cell survival was determined by the MTS assay as described in Section 2. 6-OHDA-mediated cell death is decreased even when TEMPOL was added 0.5 h after addition of 6-OHDA. The fractional cell survival of cells treated with vehicle 1 h before 6-OHDA treatment differs from each of that of each of the other treatment groups, respectively, with $^{*}p < 0.005$. (C) Fractional cell survival of MN9D cell cultures treated with varying concentrations of 6-OHDA in the presence of vehicle, TEMPOL (0.15 μM), or TEMPOL + PNA (5 μl of a 20% solution per ml of medium). $^{**}p < 0.01$; $^{***}p < 0.005$, relative to vehicle control.

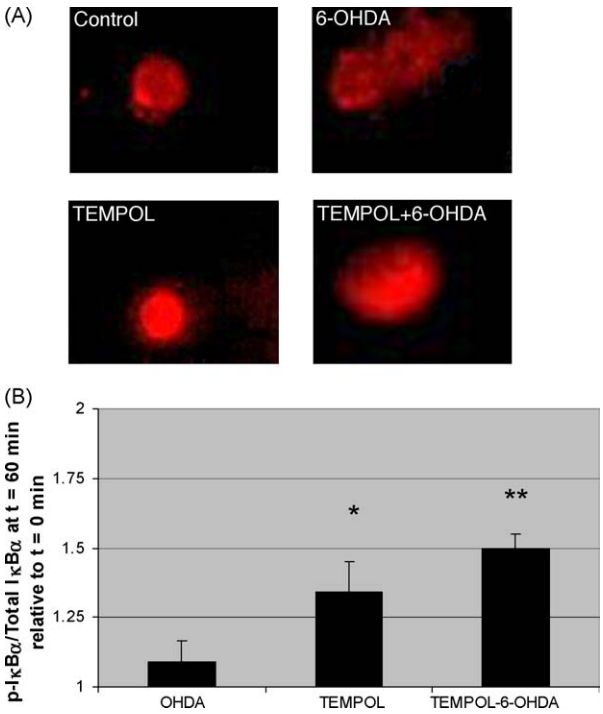

Fig. 2. (A) TEMPOL-induced nuclear translocation of the p65 subunit of NF-κB in MN9D cells. Nuclear translocation of the p65 subunit of NF-κB was visualized by immunoflurescence microscopy using a rabbit polyclonal antibody to p65 with a Cy3 goat anti-rabbit secondary antibody. Cells were processed for microscopy 60 min after initiation of treatment with TEMPOL followed by the addition of 6-OHDA or vehicle to the culture medium. (B) Densitometric analysis of Western blots for IκBα and p-IκBα (methods in [21]) demonstrates that at 60 min after treatment, MN9D cells treated with TEMPOL or TEMPOL-6-OHDA exhibit phosphorylation of IκBα while MN9D cells treated with 6-OHDA alone do not. Analogous samples studied at 10 and 180 min after treatment do not demonstrate phosphorylation of IκBα with any of the treatment regimens.

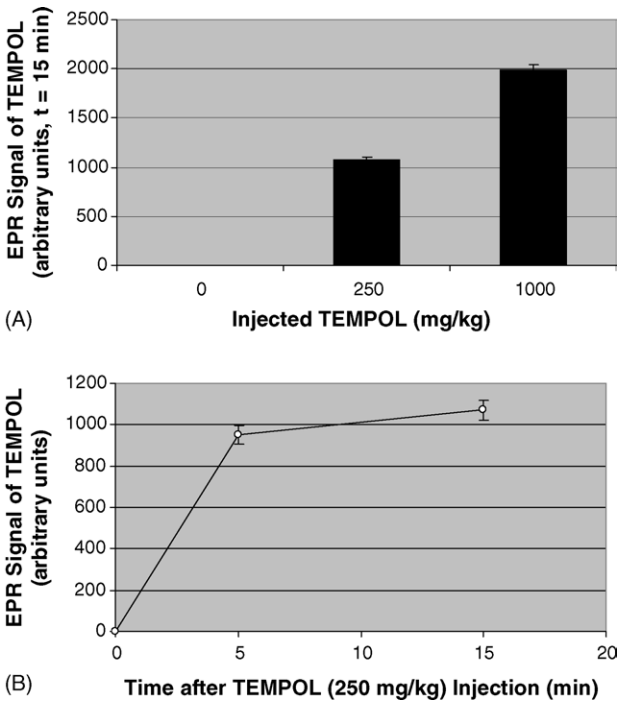

Fig. 3. TEMPOL distribution to intact brain after i.v. administration. Total (reduced + oxidized) TEMPOL was measured in mouse brain homogenates by EPR spectroscopy after addition of potassium ferricyanide as described in Section 2. Whole brain TEMPOL concentration, measured at 15 min after injection, increases in dose-dependent fashion (A). TEMPOL is detectable by EPR in intact brain within 5 min of i.v. administration to mice and plateaus (B), leaving a reservoir of available reducing potential.

concentration curve (A) and pharmacokinetics (B) of total recyclable TEMPOL (i.e., TEMPOL detectable after addition of potassium ferricyanide to brain homogenates) in the brain after i.v. administration. Although oxidation of brain TEMPOL takes place between 5 and 15 min after i.v. injection (data not shown), recyclable TEMPOL plateaus and remains as a redox-available reservoir.

3.1.4. Effects of TEMPOL on 6-OHDA-induced cell loss in the striatum and substantia nigra

6-OHDA (1 μg) was injected into the left striatum of each mouse pretreated (i.p. 40–50 min before 6-OHDA injection) with TEMPOL (200 mg/kg) or vehicle. Fig. 4 demonstrates the relative preservation of tyrosine hydroxylase-containing (TH+) cells in the 6-OHDA-treated striatum (A) and ipsilateral substantia nigra (B and C) in animals pretreated with TEMPOL. The left/right substantia nigra cell count ratio for animals treated with TEMPOL/6-OHDA differs from that for animals treated with vehicle/6-OHDA with a p maximally 0.02.

3.1.5. Effects of TEMPOL and TEMPOL-PNA on 6-OHDA-induced loss of central catecholamine metabolites in the striatum

6-OHDA-induced loss of TH+ cells in the striatum would be expected to be accompanied by loss of catecholamine metabolites in this region. Conversely, preservation of such cells by TEMPOL should be accompanied by preservation of catecholamine metabolites. Fig. 5 demonstrates that TEMPOL pretreatment (200 mg/kg i.p.) protects against 6-OHDA-induced loss of DOPAC and HVA ($p < 0.005$). The left/right striatum ratio for dopamine content was lower in eight of the 10 6-OHDA-treated animals than in any of the 10 TEMPOL/6-OHDA-treated animals; however, the difference in mean left/right dopamine content ratio between 6-OHDA- and TEMPOL/6-OHDA-treated striata did not reach statistical significance. Note that 6-OHDA treatment alone depresses DOPAC and HVA levels much more than dopamine levels; the L–R ratio for striatal dopamine (0.75) is in fact higher after TEMPOL treatment than is the analogous ratio for DOPAC (0.65) or HVA (0.65).

Fig. 5 also demonstrates that PNA enhances the protective effects of TEMPOL in this system.

3.1.6. Effects of TEMPOL on 6-OHDA-induced central dopaminergic dysfunction

Fig. 6 demonstrates that pretreatment of mice with TEMPOL (200 mg/kg i.p., 40–50 min before 6-OHDA

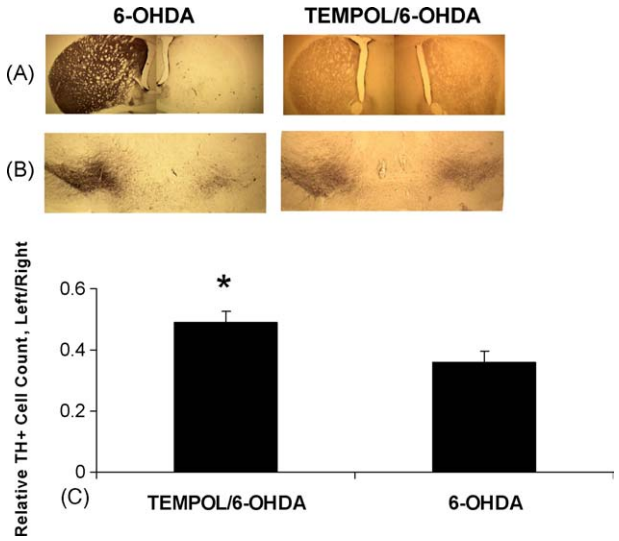

Fig. 4. Effects of TEMPOL on 6-OHDA-induced cell loss in the substantia nigra and striatum of mice. 6-OHDA (1 μg) was delivered into the left striatum 40–50 min after TEMPOL administration (200 mg/kg i.p.). Mice were sacrificed by CO_2 narcosis 1 week later. After perfusion with iced saline and 4% paraformaldehyde, the brains were taken out and sectioned using a microtone. Sections through the striatum (A) and the substantia nigra (B) were collected and every fifth slice was stained for tyrosine hydroxylase (TH), the rate-limiting enzyme for dopamine synthesis. (C) The number of TH-positive cells in the substantia nigra was determined individually for the left (injected) and right (uninjected) sides. The ratio of cell number on the left to cell number on the right decreases as increasing damage is done by 6-OHDA to the substantia nigra. TEMPOL pretreatment increases the left/right cell number ratio, and therefore, decreases the damage done by 6-OHDA. *The error bars show the S.E.M. and the ratio for TEMPOL/6-OHDA differs from that for 6-OHDA alone with $p < 0.02$.

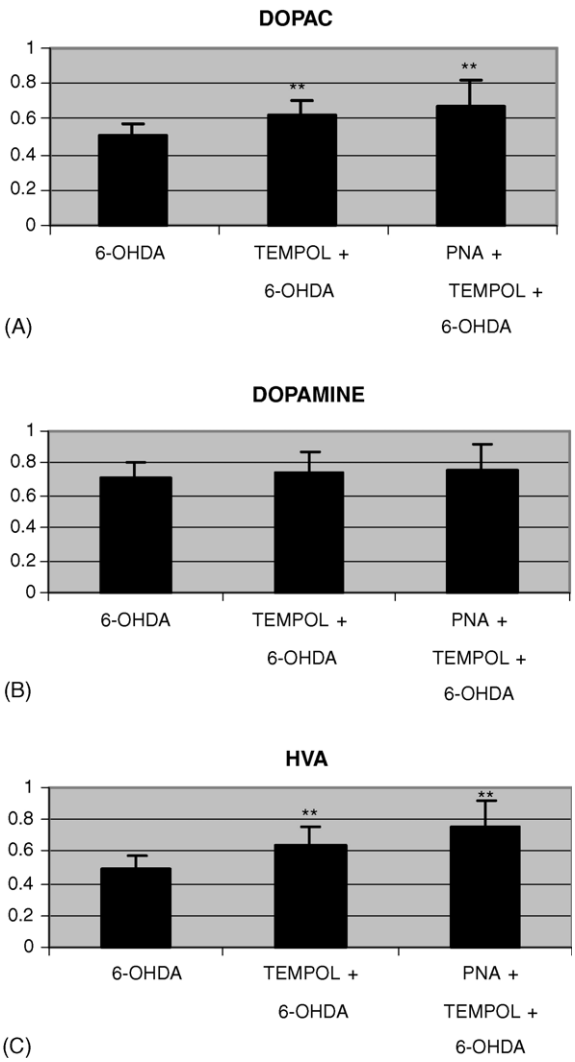

Fig. 5. Effects of TEMPOL on the levels of DOPAC, dopamine and HVA in the striatum in 6-OHDA-treated (intrastriatal injection) mice. TEMPOL (250 mg/kg, i.p.) or TEMPOL plus PNA (2 ml/kg of a 20% solution) was administered, followed by 6-OHDA (1 μg) administration into the left striatum 1 h later. One week after 6-OHDA injection, mice were sacrificed by CO_2 narcosis. The brain was removed and the striatum was dissected for measurement of the tissue concentrations of DOPAC, dopamine, and HVA by HPLC as described in Section 2. Data are presented as mean ± S.E.M. of the ratio of the concentration in the left striatum (i.e., 6-OHDA-treated) to the concentration in the right striatum (untreated). **$p < 0.01$ comparing 6-OHDA- to TEMPOL/6-OHDA-treated animals and comparing TEMPOL/6-OHDA-treated animals to TEMPOL-PNA/6-OHDA-treated animals.

treatment) reduces the effects of 6-OHDA (6 μg into the left striatum) on the rotatory behavior of mice administered D-amphetamine (2.5 mg/kg i.p.). Damage to the left striatum induced by 6-OHDA results in preferential turning of D-amphetamine-treated mice to the left. This preference is reduced by pretreatment with TEMPOL ($p < 0.0005$).

3.2. TEMPOL in in vivo models of the peripheral autonomic nervous system manifestations of Parkinson's disease

3.2.1. Effects of TEMPOL on 6-OHDA-induced peripheral dopaminergic dysfunction

Systemic (i.p.) administration of 6-OHDA to mice results in autonomic dysfunction manifested clinically by ptosis and decreased activity level [14,20,25]. As is shown in Fig. 7, TEMPOL (200 or 300 mg/kg i.p.) protects mice from ptosis (A), short-term activity decrement (B), and long-term mortality (C) associated with 6-OHDA (300 or 400 mg/kg) treatment.

In view of the protection of MN9D cells from 6-OHDA by subsequent administration of TEMPOL, mice were treated with 6-OHDA followed 20 min later by vehicle or TEMPOL treatment. Fig. 8 demonstrates the efficacy of TEMPOL administered after 6-OHDA in reducing the effects of 6-OHDA on ptosis and activity level of mice.

4. Discussion

Parkinson's disease is a neurodegenerative disease characterized primarily by selective loss of the dopaminergic neurons in the pars compacta of the substantia nigra. Although the direct cause of this selective neurodegeneration is not clearly understood, many lines of evidence have implicated reactive oxygen species in the pathogenesis of

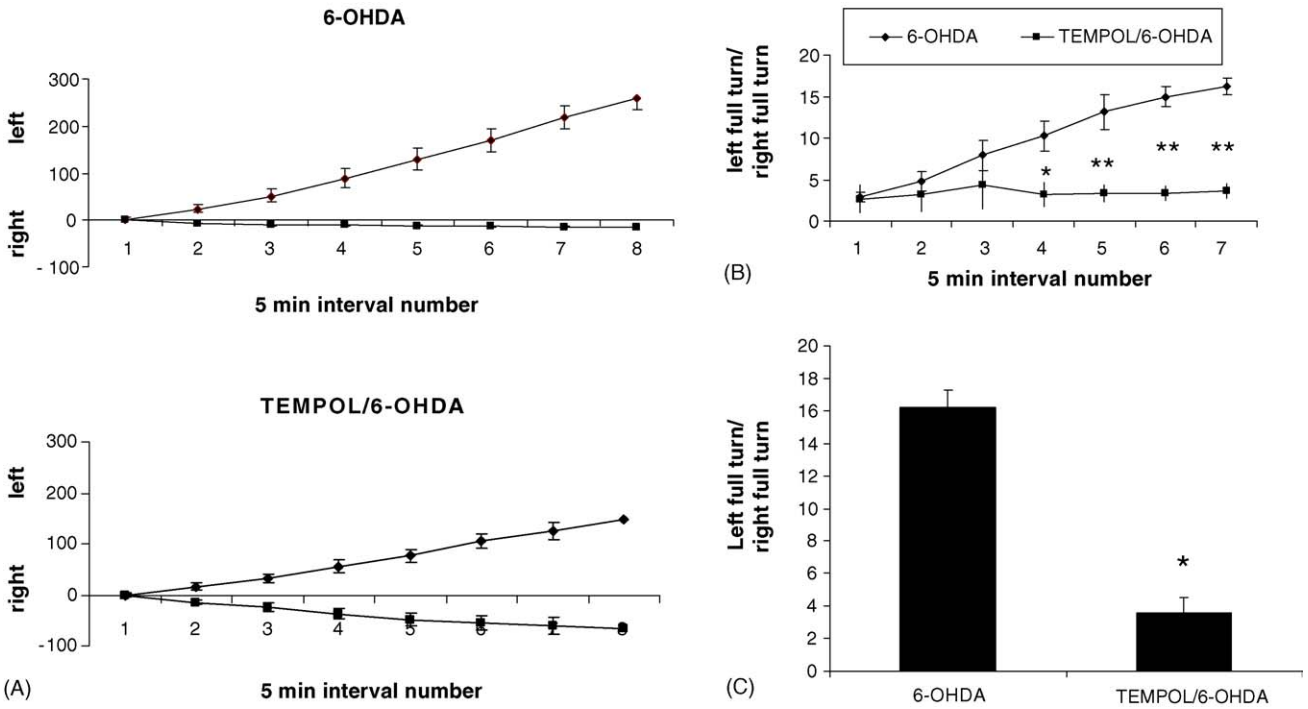

Fig. 6. Effects of TEMPOL on 6-OHDA-induced central dopaminergic dysfunction. (A) D-Amphetamine-induced rotational behavior in mice: 6-OHDA (6 μg) was delivered into the left striatum 40–50 min after TEMPOL (200 mg/kg, i.p.) injection. Six weeks later, D-amphetamine (2.5 mg/kg, i.p.) was administrated to mice to induce rotational behavior. The mice were observed during eight successive 5-min long intervals and the number of full rotations in each direction was recorded for each interval. TEMPOL-pretreated animals rotated less often to the left than non-pretreated animals. (B) The ratio of leftward full turns to rightward full turns differs significantly between TEMPOL/6-OHDA-treated and 6-OHDA-treated mice during the 16–20 min interval through the duration of the experiment. The error bars signify the S.D. $^{*}p < 0.005$; $^{**}p < 0.0005$. (C) The ratio of full leftward to full rightward rotations for the entire 35 min observation interval demonstrates four-fold less functional impairment in TEMPOL/6-OHDA-treated mice than in 6-OHDA-treated mice ($^{*}p < 0.0005$; error bars signify the S.D.).

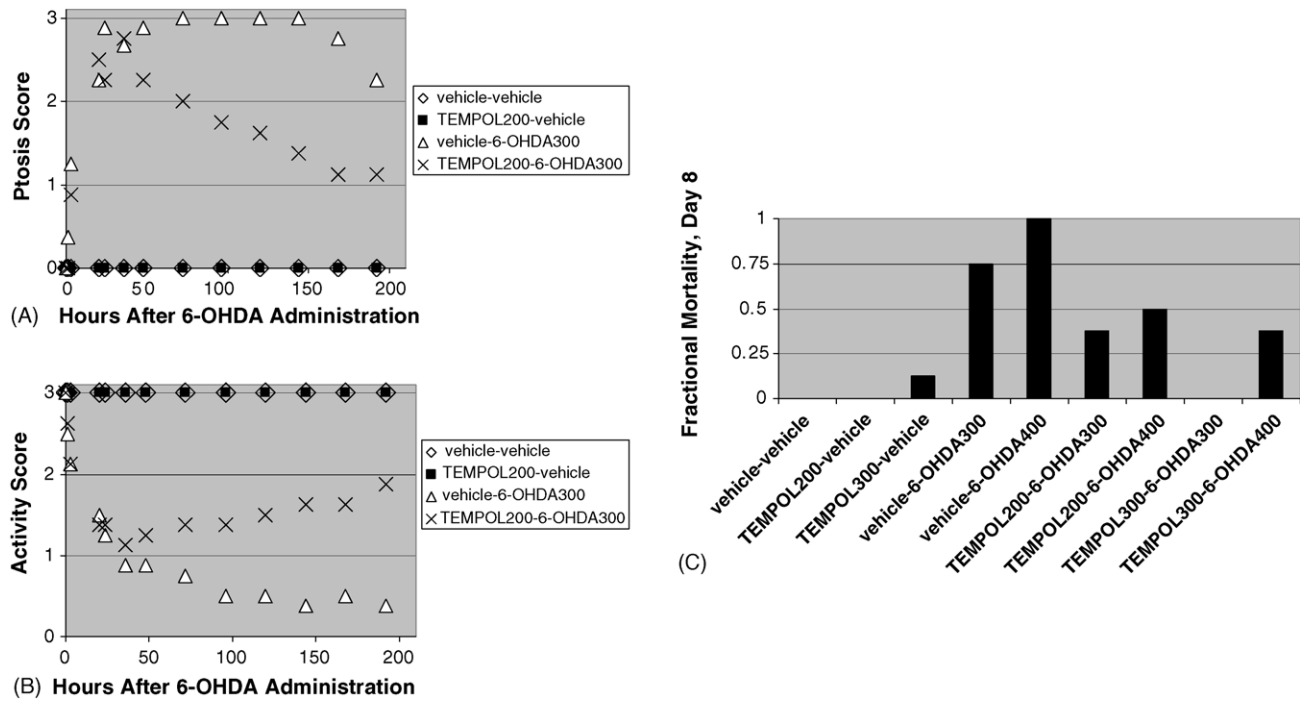

Fig. 7. Protective effects of pretreatment with TEMPOL against 6-OHDA-induced peripheral autonomic dysfunction. Autonomic dysfunction was quantitated in mice administered 6-OHDA (i.p.) after pretreatment with vehicle or TEMPOL (i.p.). The ptosis score (A), activity score (B), and mortality rate (C) of all control and experimental mice were determined, as we have previously described in detail [14,20,25]. TEMPOL or vehicle was given 10 min before administration of 6-OHDA. The difference in the ptosis and activity level between animals receiving 6-OHDA after vehicle pretreatment and animals receiving 6-OHDA after TEMPOL pretreatment was significant at all time points including and later than 36 h after 6-OHDA injection ($p < 0.05$).

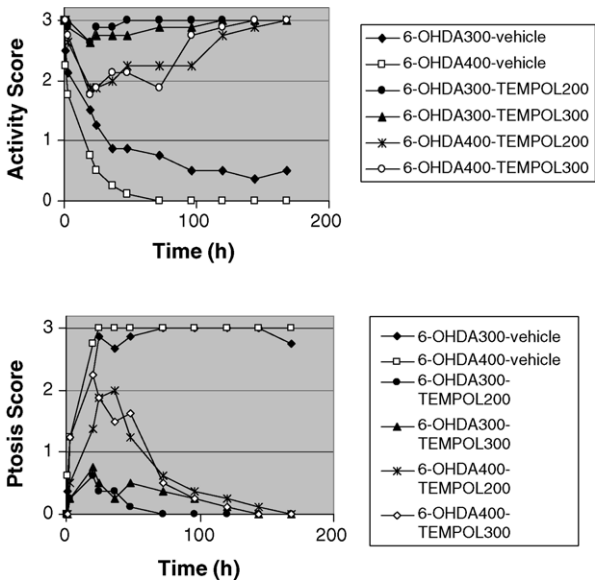

Fig. 8. Enhancement by PNA of protective effects of subsequent treatment with TEMPOL against 6-OHDA-induced peripheral autonomic dysfunction. Autonomic dysfunction was quantitated in mice administered 6-OHDA (i.p.) followed 20 min later by treatment with vehicle or TEMPOL (i.p.). The ptosis score (A) and activity score (B) of all mice were determined as we have previously described in detail [14,20,25]. The difference in the ptosis and activity level between animals receiving 6-OHDA followed by vehicle and animals receiving 6-OHDA followed by TEMPOL was significant at all time points including and later than 3 h after 6-OHDA injection ($p < 0.05$).

Parkinson's disease. The selective cell loss is thought to result from the formation of ROS-generating quinones and semiquinones and nitric oxide-generating monoamine oxidase metabolites from dopamine [15,26,27].

Dopamine can be metabolized to ROS and ROS-generating species such as hydrogen peroxide, superoxide, and hydroxyl radical. Endogenous antioxidant mechanisms, including the generation and maintenance of reduced glutathione, and the expression of antioxidant enzymes like superoxide dismutase have been demonstrated to be deficient in autopsy material from patients with Parkinson's disease [28,29]. More recent studies have demonstrated deficiencies in mitochondrial function, especially in complexes I and IV [30] proposed to be related to peroxynitrite-induced injury to mitochondrial proteins [31]. Furthermore, depletion of glutathione or interference with complex I function leads to increased α-synuclein expression [32] and fibrillar aggregation [33] in human dopaminergic neural crest cells in culture.

The implication of ROS in the pathogenesis of Parkinson's disease has led to the notion that exogenous antioxidants or strategies to increase expression of endogenous antioxidants would constitute useful therapeutic and prophylactic agents for this disease [14,34–43]. Exogenously administered coenzyme Q [30,37,38], melatonin [39], *N*-acetyl-cysteine [40,43,44], fraxetin [43], myricetin [43], and baicalein [33] have been proposed as drugs for Parkinson's disease prophylaxis and therapy. The present study adds the stable nitroxide antioxidant, TEMPOL, to this list. Unlike the other agents proposed to date, the antioxidant activity of TEMPOL is mediated by several simultaneously operational mechanisms. TEMPOL works as a superoxide dismutase mimic [18] and directly reacts with both carbon-centered and peroxy radicals [17]. Furthermore, TEMPOL can be adjunctively administered with PNA to generate a recycling antioxidant complex, a formulation that increases the active half-life and widens the therapeutic window of TEMPOL [14] and, as shown in the present studies, enhances the efficacy of TEMPOL.

6-OHDA has been used to generate in vitro and in vivo models with which to investigate the mechanism and potential therapy of Parkinson's disease [45]. This dopamine analogue is taken up by the plasma membrane dopamine transporter of central nervous system and autonomic nervous system neurons. Once in the cytoplasm, 6-OHDA generates ROS both via deamination by monoamine oxidase [46,47] and by its own enzyme-free autoxidation [48–50]. In vitro models of Parkinson's disease include treatment of MN9D cells (central nervous system analogues) or PC12 or SH-SY5Y cells (autonomic nervous system analogues) with 6-OHDA. Intrastriatal administration of 6-OHDA induces an animal model of central nervous system dysfunction in Parkinson's disease [51–53]. Intraperitoneal administration of 6-OHDA induces an animal model of autonomic dysfunction in Parkinson's disease [14,20,25,54,55].

Cell death induced in vitro by 6-OHDA has previously been shown in PC12 pheochromocytoma cells to be apoptotic in nature [56]. Although the MTS assay is often referred to as a "proliferation assay", what it measures directly is mitochondrial biochemical integrity, a measure of cell viability. As the manufacturer's package insert (Promega, Madison, WI, USA) states, "The CellTiter 96® AQ_{ueous} Assay uses the novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and the electron coupling reagent, phenazine methosulfate... MTS is chemically reduced by cells into formazan, which is soluble in tissue culture medium. The measurement of the absorbance of the formazan can be carried out using 96 well microplates at 492 nm. The assay measures dehydrogenase enzyme activity found in metabolically active cells. Since the production of formazan is proportional to the number of living cells, the intensity of the produced color is a good indication of the viability of the cells."

Our previous studies demonstrated that TEMPOL protects PC12 cells from 6-OHDA-induced apoptosis and A/J mice from 6-OHDA-induced sympathetic nervous system dysfunction [14,20]. We now demonstrate that parenterally administered TEMPOL protects mice, not only from the systemic and autonomic nervous system effects of parenterally administered 6-OHDA, but also from the central nervous system effects of intrastriatally administered 6-OHDA. The delivery of TEMPOL to the brain is not

purely a function of disruption of the blood–brain barrier; TEMPOL is delivered to the intact brain after parenteral administration. Intraperitoneal TEMPOL protects mice from cell and dopamine metabolite loss induced by intrastriatal administration of 6-OHDA. The fact that 6-OHDA treatment depresses DOPAC and HVA levels more than dopamine levels is most likely related to the fact that 6-OHDA paralyzes the metabolic machinery of those neurons that take it up at lower doses than those at which and before it paralyzes release of already synthesized and packaged dopamine. Furthermore, the half-life of intravesicular dopamine is likely longer than that of extracellular and extravesicular DOPAC and HVA, and previously synthesized dopamine may well be detectable long after 6-OHDA has destroyed the cell's ability to make DOPAC and HVA and long after previously synthesized DOPAC and HVA have disappeared. In addition, 6-OHDA alone depresses DOPAC and HVA levels much more than dopamine levels, most likely because only extravesicular dopamine is available to 6-OHDA for oxidation. This reduces the fraction of ''protected'' dopamine relative to that of DOPAC or HVA.

The effects of intrastriatal 6-OHDA on motor behavior induced by amphetamine, effects that depend upon unilateral disruption of the normal dopaminergic extrapyramidal tracts, are attenuated by TEMPOL as well. Perhaps of greatest clinical significance, the present studies demonstrate both that PNA enhances the effectiveness of TEMPOL in the central and peripheral nervous system and that TEMPOL is effective even when administered after the administration of 6-OHDA to mice.

Mechanistic studies have indicated that sequential activation of JNK and caspases follows 6-OHDA-treatment and precedes apoptosis of dopaminergic cells in culture [40,57]. Endogenous proteins the upregulation of which prevents these phenomena include heat shock protein family member, heme oxygenase-1 [30] and parkin [57]. Our prior studies demonstrated that activation and nuclear translocation of NF-κB follows TEMPOL treatment of PC12 cells and correlates with prevention of apoptosis in these cells [14]. The present studies demonstrate this phenomenon, by showing both nuclear translocation of NF-κB and phosphorylation of IκBα in MN9D cells. Activation of NF-κB has been seen as either a pro- or an anti-apoptotic event in other neural systems [58].

While antioxidants like TEMPOL may ultimately prove useful in arresting progression of Parkinson's disease, a recent study suggests that there are other mechanisms by which 6-OHDA induces apoptosis in human neural crest cells [58]. In SH-SY5Y dopaminergic neuroblastoma cells, 6-OHDA treatment results in hyperphosphorylation of glycogen synthase kinase-3-β at tyrosine 216, prompting downregulation of cyclin D1, upregulation of GADD153, and activation of procaspase-12, procaspase-3, and poly-(ADP-ribose) polymerase, and consequent apoptosis [59]. Although antioxidants were neuroprotective in this model, they did not inhibit the hyperphosphorylation of glycogen synthase kinase-3-β, implying that 6-OHDA triggers a pro-apoptotic signal transduction pathway by a mechanism separate and distinct from its generation of ROS. Antioxidants may, therefore, play an adjunctive, rather than single agent, role in the therapy of Parkinson's disease. Finally, while TEMPOL alone protects the brain and autonomic nervous system from acute oxidative injury, chronic oxidative stress would require that TEMPOL remain available and recyclable to a reducing agent. The plateau of redox-available TEMPOL in brain and the ability of PNA to recycle TEMPOL and maintain a steady-state tissue concentration of reduced, ''active'' TEMPOL [14], thereby increasing the efficacy and effective half-life of TEMPOL, make TEMPOL-PNA an attractive therapeutic candidate for Parkinson's disease and pathophysioloigcally related disorders.

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