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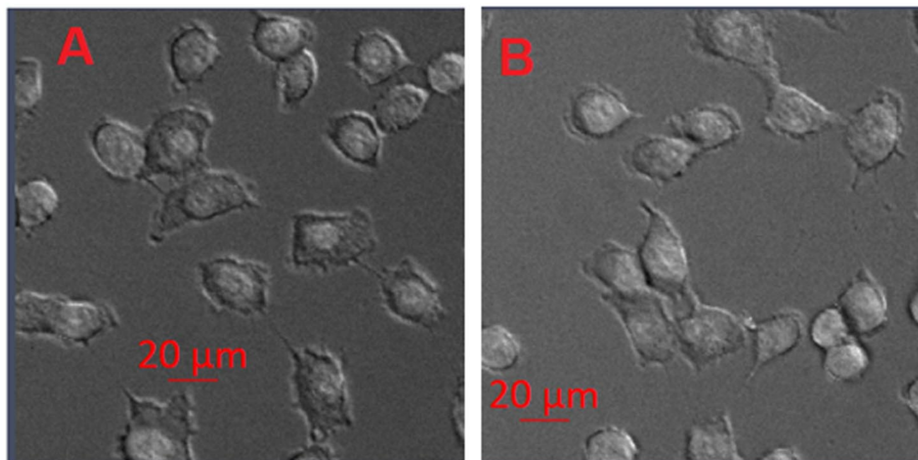

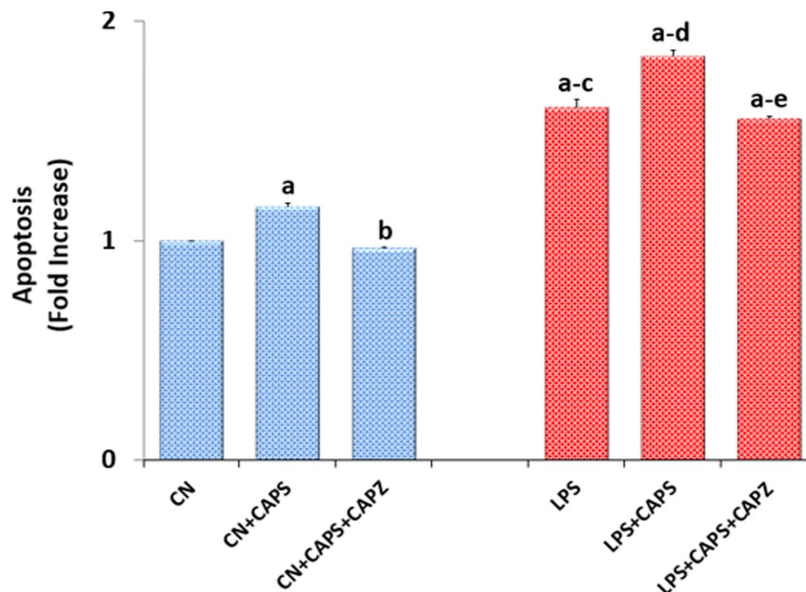

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Areas of particular interest are four topics. They are;

A-Ion Channels (Na^+- K^+ Channels, Cl^- channels, Ca^{2+} channels, ADP-Ribose and metabolism of NAD^+, Patch-Clamp applications)

B-Oxidative Stress (Antioxidant vitamins, antioxidant enzymes, metabolism of nitric oxide, oxidative stress, biophysics, biochemistry and physiology of free oxygen radicals)

C-Interaction Between Oxidative Stress and Ion Channels in Neuroscience
(Effects of the oxidative stress on the activation of the voltage sensitive cation channels, effect of ADP-Ribose and NAD^+ on activation of the cation channels which are sensitive to voltage, effect of the oxidative stress on activation of the TRP channels in neurodegenerative diseases such Parkinson's and Alzheimer's diseases)

D-Gene and Oxidative Stress
(Gene abnormalities. Interaction between gene and free radicals. Gene anomalies and iron. Role of radiation and cancer on gene polymorphism)

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RESEARCH ARTICLE

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Ginkgolide B ameliorates MPTP-induced neuroinflammation and neurodegeneration by improving mitochondrial electron transport chain complex I

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List of Abbreviations;

BSA, *bovine serum albumin;* ***CAT,*** *catalase;* ***DAT,*** *dopamine transporter;* ***EDTA,*** *ethylene diamine tetraacetic acid;* ***EGTA,*** *ethylene glycol tetraacetic acid;* ***ETC,*** *electron transport chain;* ***GPx,*** *glutathione peroxidase;* ***GR,*** *glutathione reductase;* ***GSH,*** *reduced glutathione;* ***H_2O_2,*** *hydrogen peroxide;* ***IL-1β,*** *interleukin-1-beta;* ***MAO,*** *monoamine oxidase;* ***MCP-1,*** *monocyte chemoattractant protein;* ***MDA,*** *malondialdehyde;* ***MPO,*** *myeloperoxidase;* *MPP^+, 1-methyl-4-phenyl pyridinium;* ***MPTP,*** *1-methyl-4-phenyl-1,2,3,6-tetra hydro pyridine;* ***NADPH,*** *nicotinamide adenine dinucleotide phosphate hydrogen;* ***NLRP3,*** *NLR family pyrin domain containing 3;* ***PCR,*** *polymerase chain reaction;* ***PD,*** *Parkinson's disease;* ***RIPA,*** *radioimmunoprecipitation assay buffer;* ***ROS,*** *reactive oxygen species;* ***SN,*** *substantia nigra;* ***SOD,*** *superoxide dismutase;* ***TBA,*** *thiobarbituric acid;* ***TCA,*** *trichloroacetic acid;* ***TMP,*** *1,1,3,3-tetra methoxy propane.*

Abstract

Although the pathology and clinical symptoms of Parkinson's disease (PD) are well-defined, the cellular and molecular mechanisms underlying selective degeneration of dopaminergic neurons remain unclear. Mitochondrial dysfunction and neuroinflammation are increasingly recognized as central contributors to PD pathogenesis. The leaf extract of Ginkgolide, Ginkgo biloba (L), is known for its neuroprotective properties in several neurodegenerative diseases. In the present study, we sought to investigate the neuroprotective mechanism of Ginkgolide B (BN52021) in an animal model of PD. Adult C57BL/6 mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, 30 mg/ kg b.wt.) for 5 consecutive days exhibited significant motor deficits, ameliorated by cotreatment with BN52021 (20 mg/ Kg b.wt.), as evidenced by improved motor behaviors. MPTP administration resulted in a marked reduction in the mitochondrial complex-I activity and antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase, and reductases), specifically in the substantia nigra, whereas the cortex remained unaffected. Notably, BN52021 cotreatment restored complex-I function and antioxidant enzymes in the substantia nigra, highlighting its neuroprotective properties. Additionally, MPTP exposure significantly increased myeloperoxidase activity, an oxidative stress and inflammation marker mitigated by BN52021. Moreover, the inflammatory markers, Nod-like receptor family pyrin domain containing 3, Monocyte chemoattractant protein-1, and pro-interleukin-1β were significantly upregulated following MPTP administration, indicating the activation of the

inflammasome pathway. However, coadministration of MPTP with BN52021 effectively suppressed the upregulation of these inflammatory markers, suggesting a strong anti-inflammatory effect. These findings underscore the therapeutic potential of BN52021 in PD, primarily through its ability to enhance complex-I activity, restore antioxidant defense, and suppress neuroinflammation.

Keywords; BN52021, Ginkgolide B, Inflammasome, 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine, Neuroprotection, Parkinson's disease

Introduction

Parkinson's disease (PD) is the second most prevalent progressive neurodegenerative disorder after Alzheimer's, caused by pathophysiological loss or degeneration of dopaminergic neurons in the substantia nigra of the midbrain (Yildizhan et al., 2020). PD patients typically exhibit resting tremors, rigidity, bradykinesia, and a stooped posture, among other motor and nonmotor symptoms (Jankovic, 2008; Meireles et al., 2012). Several studies have shown that mitochondrial dysfunction, oxidative stress, and neuroinflammation play an important role in the initiation and progression of neurodegeneration in PD (Yildizhan et al., 2022; Yildizhan and Naziroglu, 2022). In PD, mitochondrial dysfunction, especially at complex-I of the electron transport chain (ETC), impairs cellular energy production and triggers inflammation (Beal, 2003; Pirunkaset et al., 2024). Defective complex-I leads to increased reactive oxygen species (ROS) and the release of mitochondrial DNA into the cytosol, which can activate the NLRP3 inflammasome (Holbrook et al., 2021). This inflammasome activation promotes the release of proinflammatory cytokines like interleukin-1β (IL-1β), contributing to neuroinflammation. Chronic neuroinflammation, combined with mitochondrial dysfunction, exacerbates dopaminergic neuronal cell death in the substantia nigra, accelerating PD progression. Microglia, immune cells of the central nervous system, are densely populated in the substantia nigra and striatum, the regions of the brain that are critically affected in PD. Post-mortem studies of brain tissue from PD patients showed a significant increase in activated microglia, particularly in the midbrain region where dopaminergic neurons are localized (McGeer et al., 1988). Activated microglia contribute to the increased levels of proinflammatory and inflammatory cytokines as well as increased levels of free radicals and pro-apoptotic proteins in the substantia nigra, striatum, and cerebrospinal fluid of patients with PD (Nagatsu et al., 2000a, 2000b; Harms et al., 2021).

Microglia promote neuronal cell death by producing inflammatory mediators and interacting with α-synuclein, facilitating its propagation and aggregation or having neuroprotective effects by secreting neurotrophic factors (Sanchez-Guajardo et al., 2013; Joers et al., 2017; Yıldızhan et al., 2019). The Nod-like receptor family pyrin domain containing 3 (NLRP3) inflammasome, a multiprotein complex initiating a proinflammatory state in microglia, plays a key role in PD. In PD models, NLRP3 inflammasome activation in microglia is triggered by α-synuclein (Gordon et al., 2019), with different forms of α-synuclein eliciting specific microglial responses through NLRP3, including α-synuclein degradation (Scheiblich et al., 2021), highlighting its potential involvement in the disease. 1-Methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) is one of the neurotoxins most commonly used to establish animal models of PD (Zhang et al., 2017). MPTP is a lipophilic compound that crosses the blood-brain barrier and is converted into a mitochondrial toxicant, 1-methyl-4-phenyl pyridinium (MPP+), by monoamine oxidase in the astroglia (Russell et al., 1979). MPP+ is then taken up by dopaminergic neurons through the dopamine transporter and exerts its toxic effects by inhibiting complex-I of the mitochondrial respiratory chain, leading to oxidative stress in neuronal cells, followed by intrinsic apoptosis, neuroinflammation, excitotoxicity, and the accumulation of inclusion bodies, ultimately leading to neuronal loss in the substantia nigra (Cassarino et al., 1999; Zhu et al., 2012; Yildizhan, Cinar, et al., 2022). The activation of microglia, a defining feature of neuroinflammation and oxidative stress, causes neuronal damage, especially in neurodegenerative diseases, by releasing proinflammatory cytokines (Block et al., 2007; Long-Smith et al., 2009). Ginkgolide B (BN52021), a diterpenoid lactone derived from Ginkgo biloba (L), exhibits neuroprotective effects and holds promise for mitigating neurodegeneration (Liu et al., 2020; Wu et al., 2020; Zhao et al., 2020). It acts primarily as a potent antagonist of platelet-activating factor (PAF) receptors, thereby reducing neuroinflammation (Oberpichler et al., 1990) and oxidative stress (Gordon et al., 2019), the key contributors to neuronal damage in conditions like Alzheimer's disease (AD) and PD. Additionally, Ginkgolide B modulates mitochondrial function, prevents

apoptosis, and supports synaptic integrity, making it a valuable candidate for therapeutic interventions targeting neurodegenerative diseases. Its multi-modal mechanisms highlight its potential for preserving neuronal health and preventing disease progression. In the present study, we sought to investigate the neuroprotective mechanism of BN52021 in an animal model of PD.

Materials and Methods

Chemicals

MPTP (Cat. No. M0896), Ginkgolide B from Ginkgo biloba leaves (BN52021, Cat No. G6910), trichloroacetic acid (Cat. No. T0699), thiobarbituric acid (Cat. No. T550), hexadecyltrimethylammonium bromide (H5882), dimethyl sulfoxide (CAS 67-68-5, Cat. No. D2650), 1,1,3,3- tetramethoxypropane (Cat. No. 108383), and O-dianisidine hydrochloride (D3252) were purchased from Sigma (Sigma-Aldrich Co., St. Louis, MO, USA). The protein assay kit (Pierce™ Bradford Protein Assay Kit) was purchased from Thermo Fisher Scientific, Waltham, MA, USA). Sodium hydroxide pellets (CAS 1310-73-2, Cat. No. 106402), potassium permanganate (CAS 7722-64-7, Cat. No. 105082), sodium dihydrogen phosphate dihydrate (CAS 13472-35-0, Cat. No. 106342), and potassium chloride (CAS 7447-40-7, Cat. No. 104936) were purchased from Merck Millipore, Darmstadt, Germany. Sodium chloride (CAS 7647-14-5, Cat. No. 31721) was purchased from Sisco Research Laboratories Pvt. Ltd, Maharashtra, India. Phosphate-buffered saline (PBS), pH 7.4 (Cat. No. M1866) was obtained from Himedia Laboratories Pvt. Ltd, Maharashtra, India. The QIAGEN RNeasy Lipid Tissue Mini Kit (Cat. No. 1023539) was purchased from QIAGEN India Pvt. One-Step TB Green® PrimeScript™ RT-PCR Kit II (DSS Takara Bio India Pvt. Ltd. India). All other chemicals and consumables were purchased from locally available commercial vendors.

Animals

Forty-eight adult male C57BL/6 mice, 8-10 weeks old, weighing around 25-30 g, were purchased from Mass Biotech, Chengalpet, Chennai, and housed in clean filter-top polypropylene cages (290L×220W×140H mm) in the Central Animal House of Rajah Muthiah Medical College, Annamalai University, Chidambaram. The animals were maintained under a well-regulated light and dark cycle (12h:12h) at a controlled room temperature of 25±1° C with a relative humidity between 45 to 55%. The animals were fed standard rodent chow and water ad libitum. The animal experiments were carried out per the Guide for the Care and Use of Laboratory Animals 2011 (National Research Council of National Academies, USA). All the treatments were done under the supervision of a veterinarian at the Central Animal House of Rajah Muthiah Medical College, Chidambaram. The treatment was carried out in a single-blinded manner, where the investigators were unaware of the treatment group allocation until the end of the study. The animal experiments were approved by the Institutional Animal Ethics Committee, Rajah Muthiah Medical College, Annamalai University (Approval ID: AU-IAEC/PR/1283/10/20).

Drug preparation and groups

The standard solution of MPTP was prepared by diluting it with sterile normal saline in a sealed vial of MPTP at a concentration of 30 mg/ ml. This stock solution was then diluted depending on the working concentration determined based on the weight of the mice. All the animals were acclimatized to the laboratory conditions for one week before starting the drug administration. After acclimatization, the mice were randomly divided into the following groups and given the drugs: Group I: Vehicle control (Normal saline, i.p. injection); Group II: MPTP (30 mg/ Kg body weight, i.p. injection); Group III: BN52021 (20 mg/ Kg body weight, oral administration) and Group IV: MPTP + BN52021 (i.p. injection + oral administration). All the chemicals were dissolved in normal saline and administered/ or co-administered for five consecutive days.

The dosages of MPTP and BN52021 were selected based on their pharmacological relevance, consistency with prior research (Jackson-Lewis et al., 2007; Zhao et al., 2016), and the experimental objectives of the study. MPTP was administered at 30 mg/kg body weight intraperitoneally, a dose commonly used to model Parkinson's disease-like pathology in mice. This dosage induces progressive dopaminergic neurodegeneration in the substantia nigra without causing excessive mortality (Jackson-Lewis et al., 2007). Administering this dose for five consecutive days ensures sustained neurotoxin exposure, simulating the chronic neurodegenerative effects of PD rather than acute toxicity. For BN52021, the selected dose of 20 mg/kg body weight via oral administration aligns with preclinical evidence demonstrating its efficacy

as a platelet-activating factor (PAF) receptor antagonist (Bellizzi et al., 2016; Shao et al., 2021). This dose effectively mitigates neuroinflammation, oxidative stress, and excitotoxicity, key mechanisms involved in MPTP-induced neurotoxicity, without significant off-target effects. The oral administration route also ensures better compliance and avoids complications related to injection site irritation. The combination of these dosages and routes allows for a comprehensive evaluation of the neuroprotective efficacy of BN52021 against MPTP-induced dopaminergic neurotoxicity while maintaining experimental consistency and safety.

Motor behaviour

Before treatment and after treatment, all the mice were subjected to motor behavioral tests under dim light, and the animals exhibiting abnormal behavioral activities before treatments were excluded from the experiments.

Open field and gridline crossing test

The natural behavior and movement of mice on an open field were observed before and after treatment with MPTP in the presence or absence of BN52021, as described earlier (Chen et al., 2015; Sun et al., 2021), with slight modification. Briefly, the movement of animals was recorded for at least 5 min, and any abnormal activities, such as slow movement, dragging of hind legs, etc., were noted. Similarly, the average number of gridlines crossed by each mouse was recorded for 5 min before and after treatment with MPTP in the presence or absence of BN52021 (**Fig. 1 A**).

Grooming and rearing behavior tests

Grooming behaviors (rubbing the body with the paws or mouth or rubbing the head with the paws), rearing behaviors (standing on the hind legs), and the duration of immobility were determined (**Fig. 1B**) for 5 min as described earlier (Kelley, 2001), with slight modification. The tests were performed before and after treatment with MPTP in the presence or absence of BN52021.

Pole test

The pole test was performed as previously described (Ogawa et al., 1985; Matsuura et al., 1997) with minor modifications. Mice were placed on the top of a vertical pole (height 45 cm). The average time taken by the mice to

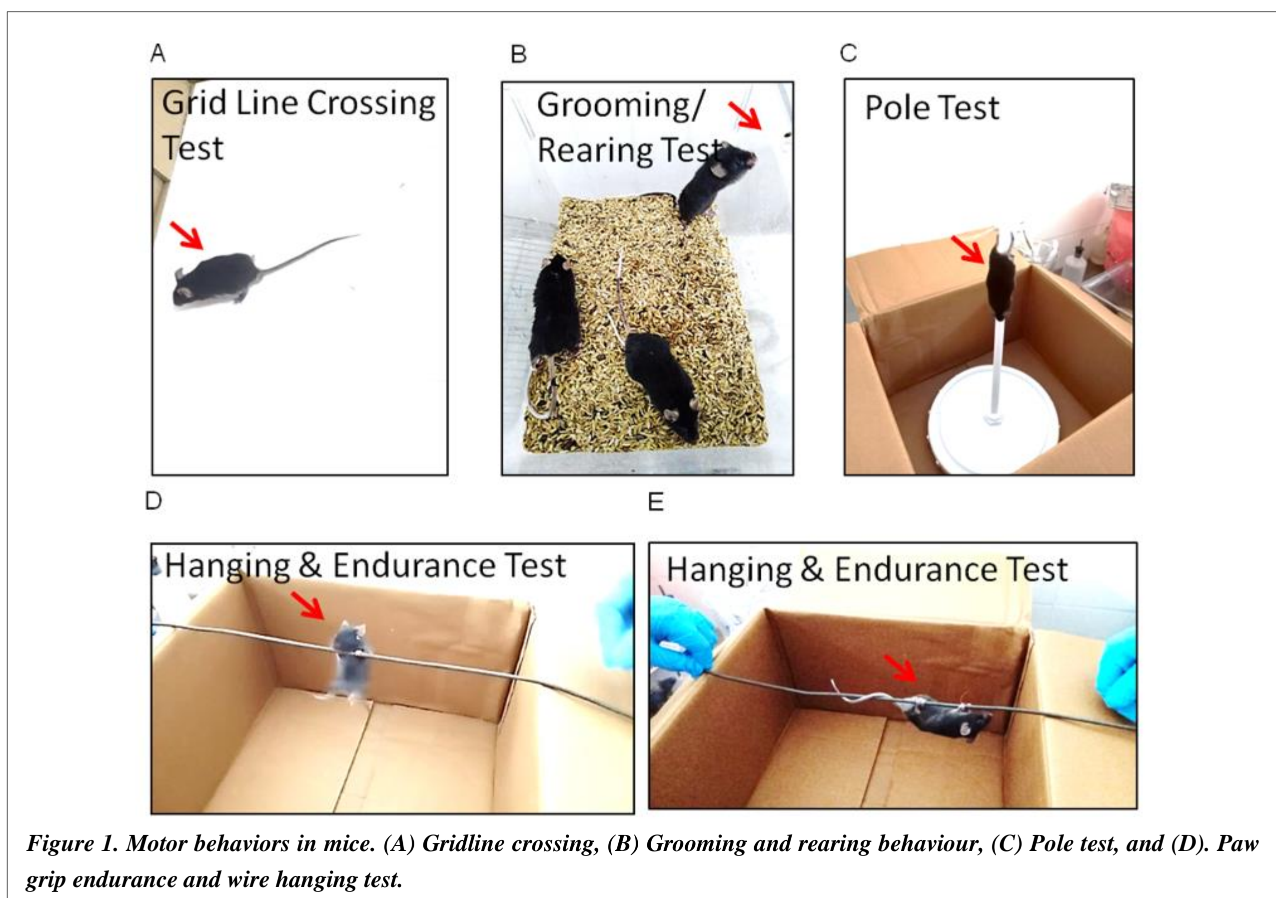

Figure 1. Motor behaviors in mice. (A) Gridline crossing, (B) Grooming and rearing behaviour, (C) Pole test, and (D). Paw grip endurance and wire hanging test.

reach the bottom of the pole and eventually touch the flat surface was recorded (**Fig. 1C**). The tests were performed before and after treatment with MPTP in the presence or absence of BN52021.

Paw grip endurance test or wire hanging test

The paw grip endurance or wire hanging test was carried out as described earlier {Heidari, 2019 #27}, with slight modification. Briefly, the mice were placed on a wire (length 50 cm, diameter 5 mm, and height 20 cm from the ground). The wire was then rotated to place the mice in a hanging state. The duration the mouse could hold itself was recorded, then the average time taken for each group was compared with that of the control group. The tests were performed before and after treatment with MPTP in the presence or absence of BN52021. Hanging tests were repeated thrice for each mouse with a 2-minute resting period (**Fig.1D & E**).

Collection of substantia nigra and cortex

A coronal section was made at the caudal portion of the cerebral cortex, approximately 2 mm anterior to the superior colliculi. The cortical remnants anterior to the superior colliculi were carefully removed, and the posterior regions were severed. The resulting tissue block, corresponding roughly to the coordinates -2.5 mm to -5.0 mm relative to the bregma, was further dissected to exclude the basal portion of the cerebral peduncles ventrally and the colliculi dorsally, isolating the substantia nigra from the ventral midbrain. Specifically, the substantia nigra from both the left and right hemispheres of each brain were pooled to ensure an adequate quantity of tissue for downstream analyses. The brain slice anterior to the initial coronal cut was used for the dissection of the cortex, and the forebrain cortex was collected using a scalpel. All dissected tissues were rapidly flash-frozen in liquid nitrogen immediately after collection.

Mitochondrial isolation

Mitochondria were isolated from the substantia nigra and cortex using a differential centrifugation method adapted earlier (Berman et al., 1999). All mitochondrial assays were performed within 24 hours of isolation, using 20 µg of mitochondrial protein for each assay. In brief, the substantia nigral tissues from the right and left brain or cortex were quickly removed, washed, and homogenized in 500 µl of isolation medium containing 225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mM EGTA, 1 mg/ml bovine serum albumin (BSA), pH 7.4, and centrifuged at 2,000 g ((Eppendorf Centrifuge 5810R, Eppendorf India Pvt. Ltd. India) for 3 minutes. The resulting pellets were resuspended in 300 µl of isolation medium and subjected to a second centrifugation at 2,000 g. The supernatants were pooled and centrifuged at 10,000 g for 10 minutes, and an isolation medium containing 0.02% digitonin was then centrifuged at 12,000 g for 10 minutes. The brown mitochondrial pellets, free of the synaptosomal layer, were resuspended in 500 µl of medium and centrifuged again at 12,000 g for 10 minutes. The final mitochondrial pellets were resuspended in 200 µl of the medium. Mitochondrial protein concentrations were determined using the Pierce™ Bradford Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Mitochondrial complex activities

The complex I, II, and IV enzymatic activities were carried out using the standard protocol (Spinazzi et al., 2012). For assessing the enzymatic activity of complex I (NADH dehydrogenase), the reaction mixture contained 20 µg of mitochondrial protein in 100 µl of 0.5 M potassium phosphate buffer (pH 7.5), 30 µl of KCN (10 mM), 60 µl of fatty acid-free BSA (50 mg/ ml), and 10 µl of 10 mM NADH After thorough mixing, the absorbance was measured at 340 nm for 3 minutes. The extinction coefficient of NADH at 340 nm is 6.2 $mM^{-1}cm^{-1}$. The mitochondrial complex I activity was expressed as nmol of NADH oxidized/ min/mg protein.

Activities of antioxidant enzymes and lipid peroxidation

Superoxide dismutase

The activity of superoxide dismutase (EC.1.15.1.1) was assessed according to the method described earlier (Marklund et al., 1974). The assay mixture consisted of 240 µl of 50 mM Tris-HCl buffer containing 1 mM EDTA (pH 7.6), 30 µl of 0.2 mM pyrogallol, and 10 µl of the substantia nigral or cortical tissue homogenate. The increase in absorbance was measured at 10-second intervals for 3 minutes at 420 nm against a blank containing all components except the enzyme and pyrogallol using a spectrophotometer (SpectraMax i3x). The molar extinction coefficient used to calculate enzyme activity expressed as nmol of pyrogallol oxidized/ min/ mg protein was 6.22 x 10^3 $M^{-1}cm^{-1}$.

Catalase

Catalase (EC.1.11.1.6) activity was assessed following the protocol outlined before (Claiborne, 1985). The assay mixture comprised 240 µl of phosphate buffer (0.05 M, pH 7.0), 1 µl of 19 mM hydrogen peroxide, and 5 µl of tissue homogenate. The decrease in absorbance was measured at 10-second intervals for 5 minutes at 240 nm against a blank containing all components except the tissue homogenate using a spectrophotometer (SpectraMax i3x). The molar extinction coefficient used for calculating the catalase activity, expressed as µM H_2O_2 consumed/ min/mg protein, was 43.6 $M^{-1}cm^{-1}$.

Glutathione peroxidase

Glutathione peroxidase (EC.1.11.1.9) activity was determined following the procedure outlined earlier (Mohandas et al., 1984). The assay mixture comprised 159 µl of phosphate buffer (100 mM, pH 7.6), 10 µl of 10 mM EDTA, 10 µl of sodium azide, 50 µl of glutathione reductase, 10 µl of reduced glutathione, 10 µl of 200 mM NADPH, 1 µl of hydrogen peroxide, and 1 µl of the substantia nigral or cortical tissue homogenate. The decrease in absorbance due to NADPH consumption was measured at 10-second intervals for 3 minutes at 340 nm against a blank containing all components except the enzyme using a spectrophotometer (SpectraMax i3x). The molar extinction coefficient used for calculating the glutathione peroxidase activity expressed as nmol of GSH oxidized/ min/ mg protein was 6.22 x 10^3 $M^{-1}cm^{-1}$.

Glutathione reductase

Glutathione reductase (EC.1.6.4.2) activity was determined according to the method outlined before.(Carlberg et al., 1975) The assay mixture consisted of 175µlof phosphate buffer (100 mM, pH 7.6), 10 µl of 200 mM NADPH, 10 µl of 10 mM EDTA, 5 µl of 20 mM oxidized glutathione, and 5 µl of the substantia nigral or cortical tissue homogenate. The decrease in absorbance due to NADPH consumption was measured at 10-second intervals for 3 minutes at 340 nm against a blank containing all components except the enzyme using a spectrophotometer (SpectraMax i3x). The molar extinction coefficient used for calculating the glutathione reductase activity expressed as nmol of GSH reduced/ min/ mg protein was 6.22 x 10^3 $M^{-1}cm^{-1}$.

Lipid peroxidation

Lipid peroxidation was assessed by measuring thiobarbituric acid (TBA) reactive substances and quantifying malondialdehyde (MDA) with 1,1,3,3-tetramethoxypropane as the standard. Briefly, 10% of substantia nigra and cortical tissue homogenate in RIPA buffer was centrifuged, and a portion of the supernatant-containing protein was precipitated using trichloroacetic acid and centrifuged at 3,000 rpm for 10 min 4 °C. A 200 µL aliquot of the supernatant was mixed with 200 µL of 0.67% TBA and incubated for 30 min in a boiling water bath (100 °C). The contents were cooled to room temperature, and the absorbance was read at 532 nm (SpectraMax i3x). The concentration of thiobarbituric acid reactive substances was calculated using a standard curve, and the results were expressed as nmol of TBARS /mg protein (Maniradhan et al., 2024).

Neuroinflammatory markers

Myeloperoxidase activity

MPO activity was assessed in the substantia nigra and cortex to find out neuroinflammation, following the method as described earlier (Barone et al., 1991) with a slight modification. The substantia nigral or cortical tissue was homogenized in 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide, followed by centrifugation at 10,000 g (Eppendorf Centrifuge 5810R, Eppendorf India Pvt. Ltd. India) for 20 minutes at 4 °C. An equal volume of the resulting supernatant or standard was mixed with a PBS solution containing O-dianisidine hydrochloride and 0.0005% H_2O_2. After 5 minutes, the reaction was terminated by adding 1.2 M hydrochloric acid, and the change in optical density was measured spectrophotometrically at 460 nm. MPO activity was expressed in milliunits (mU)/ mg tissue.

NLRP3, Pro-IL-1β and MCP-1 expressions

Total RNA was isolated from substantia nigra using QIAGEN RNeasy Lipid Assay Tissue Mini Kit and quantified at 260 nm (Genova Nano Micro-volume Spectrophotometer). The RNA recovered from substantia nigra was used to perform the reverse transcription using One Step TB Green Prime Script RT-PCR Kit II (Cat. No. RR086A, DSS Takara Bio Pvt. Ltd. Delhi, India). RT-PCR was carried out in duplicates in a 10 µL reaction volume containing 1 µL RNA, 9 µL TB green master mix, and 0.4

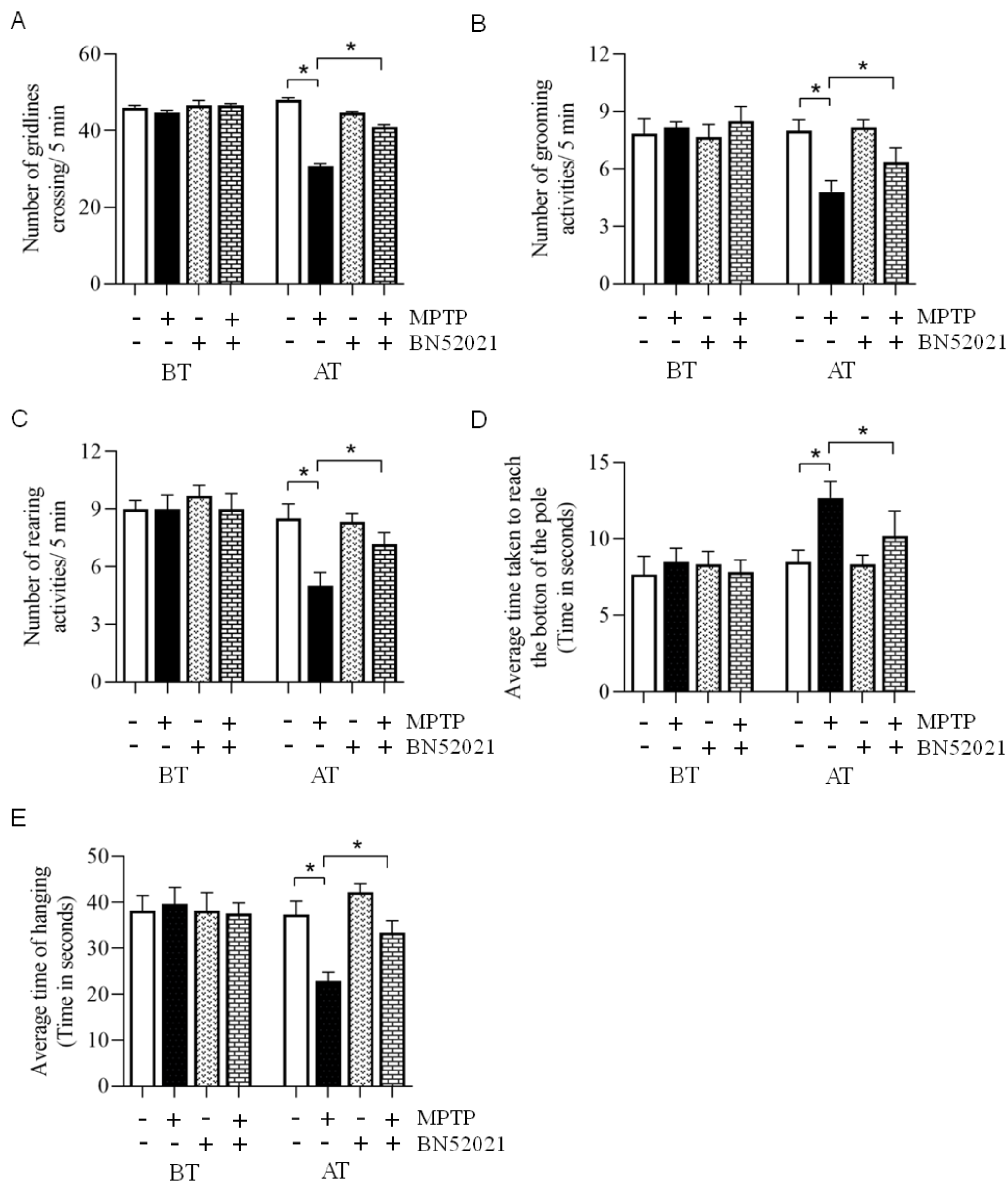

***Figure 2. (A-E).** MPTP induces motor dysfunction in mice. A. Grid line crossing. MPTP administration significantly decreased the number of gridlines crossed by the mice compared to the control (p<0.05, n=6), and coadministration of MPTP and BN52021 improved the gridline crossing compared to MPTP alone (p<0.05, n=6). (B-C). Grooming and Rearing activities. The grooming activities decreased significantly following MPTP administration compared to control or with BN52021 (p<0.05, n=6). (D). Pole test. After MPTP administration, the average time to reach the bottom of the pole increased significantly compared to control or cotreatment with BN52021 (p<0.05, n=6). (E). Wire hanging test. After MPTP administration, the average wire hanging time decreased significantly compared to control or coadministration of MPTP and BN52021 (p<0.05, n=6). The asterisk represents significant changes against the control or MPTP group.*

μL of 10 μM of respective primer. The primer sequence for NLRP3 (forward 5'-ACTTTGTGCAGAGTGCCATG-3' and reverse 5'- AACCCAGGGAAAGCGTTTTG -3'), Pro-IL-1β (forward 5'-GAAATGCCACCTTTTGACAGTG-3' and reverse 5'-CTGGATGCTCTCATCAGGACA-3'), MCP-1 (forward 5'-TTAAAAACCTGGATCGGAACCAA-3' and reverse 5'-GCATTAGCTTCAGATTTACGGGT-3') and 18s RNA (forward 5'- ATCCTGCCAGGTAGCATATGC-3' and reverse 5'-TGAGCCATTCGCAGTTTCAC-3') were obtained from the Eurofins Genomics India Pvt. Ltd, India. The amplification conditions consisted of cDNA synthesis (42 °C for 5 min) and initial denaturation (95 °C for 10 min), followed by 40 cycles of 95 °C for 5s and 58 °C for

30s (annealing and extension). The quantification was performed using the delta-delta Ct method (Schmittgen et al., 2008).

Statistical analysis

All the data were analyzed using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA). The statistical analyses were carried out by Oneway ANOVA followed by Tukey's multiple comparison test, and the results were expressed as mean±SEM. The p values <0.05 were considered statistically significant.

Results

Motor behavior

In the open field gridline crossing test, the average number of gridlines crossed by the mice treated with MPTP was significantly reduced (p<0.05, n=6) compared to the control group (**Fig. 2A**) as well as before treatment (BT). These locomotor deficits, characterized by slow movement and hind leg dragging, resembled PD-like symptoms. However, coadministration of MPTP and BN52021 significantly improved motor activities compared to the MPTP-treated group alone (p<0.05, n=6). The mice showed increased mobility, reduced hind leg dragging, and movement patterns similar to those observed in the control group. Notably, treatment with BN52021 alone did not

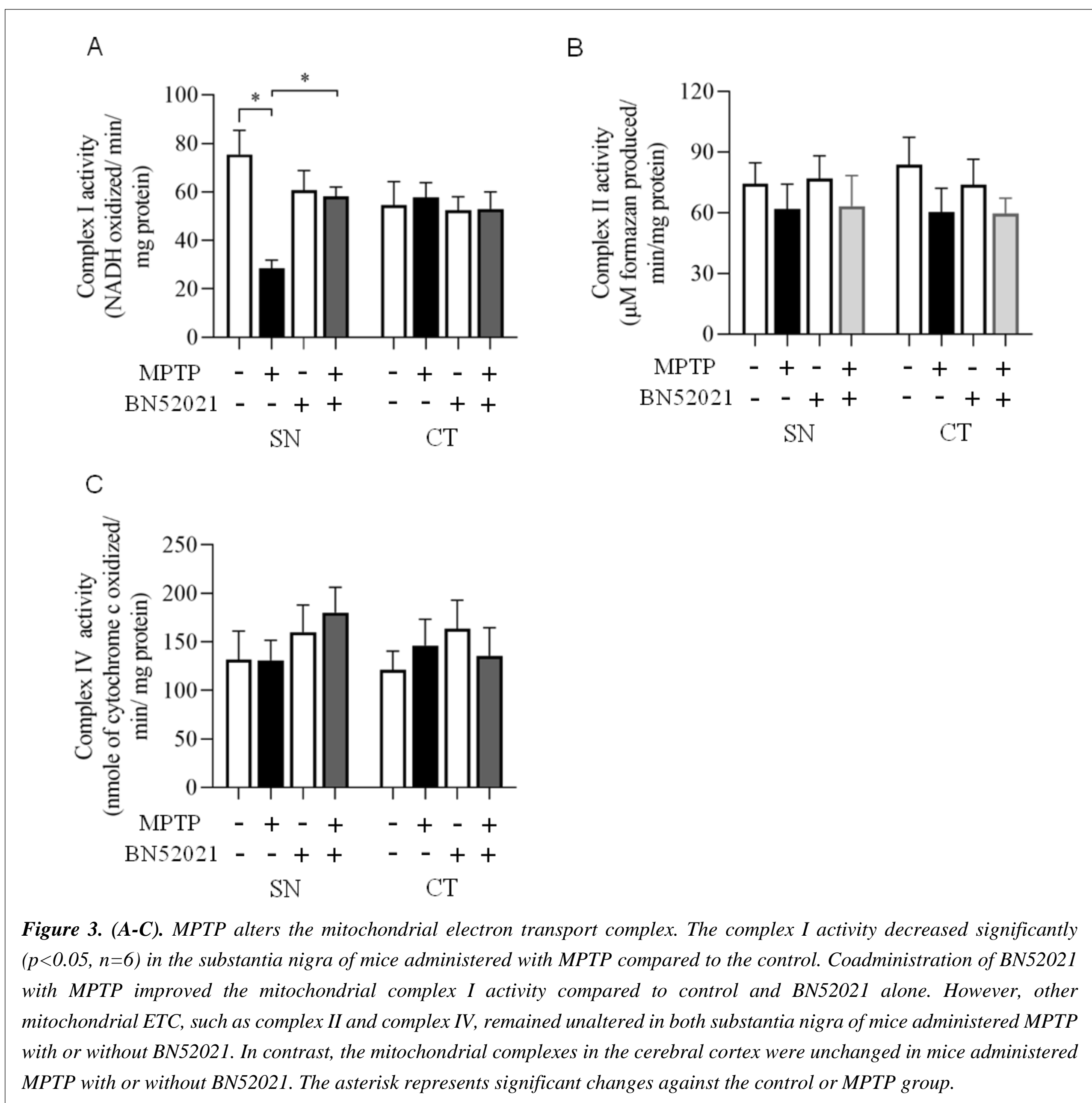

Figure 3. (A-C). MPTP alters the mitochondrial electron transport complex. The complex I activity decreased significantly (p<0.05, n=6) in the substantia nigra of mice administered with MPTP compared to the control. Coadministration of BN52021 with MPTP improved the mitochondrial complex I activity compared to control and BN52021 alone. However, other mitochondrial ETC, such as complex II and complex IV, remained unaltered in both substantia nigra of mice administered MPTP with or without BN52021. In contrast, the mitochondrial complexes in the cerebral cortex were unchanged in mice administered MPTP with or without BN52021. The asterisk represents significant changes against the control or MPTP group.

elicit any significant changes in locomotor activities compared to the control group (**Fig. 2A**). MPTP-treated mice also exhibited a significant reduction in grooming behaviors, including decreased body and head rubbing,

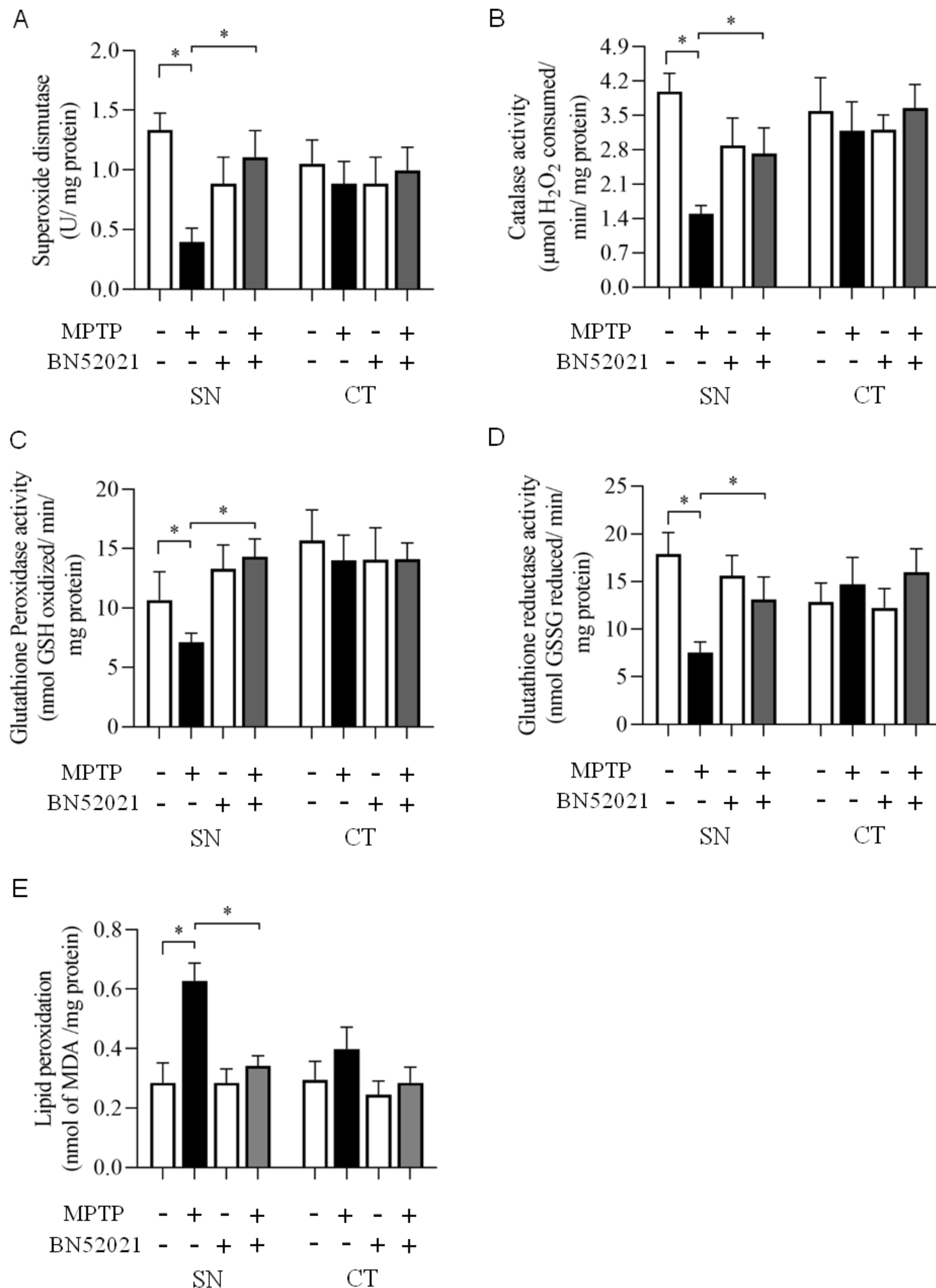

Figure 4. (A-E). *MPTP alters the antioxidant enzyme activities and lipid peroxidation. The enzymatic activities of superoxide dismutase (A), catalase (B), glutathione peroxidase (C), and glutathione reductase (D) decreased significantly in substantia nigra of mice administered with MPTP compared to control ($p<0.05$, n=6). However, coadministration of MPTP and BN52021 significantly ameliorated the MPTP-induced reduction in the antioxidant enzyme activities in substantia nigra compared to MPTP alone ($p<0.05$, n=6). The lipid peroxidation (E) levels increased significantly following MPTP administration compared to control, whereas coadministration of BN52021 with MPTP completely ameliorated the increased lipid peroxidation levels ($p<0.05$, n=6). In contrast, the mitochondrial complexes in the cerebral cortex (CT) were unchanged in mice administered MPTP with or without BN52021. The asterisk represents significant changes against the control or MPTP group.*

compared to the control group (p<0.05, n=6) (**Fig. 2B**) as well as before treatment (BT). However, coadministration of MPTP and BN52021 significantly alleviated these abnormal grooming behaviors compared to the MPTP-treated group (p<0.05, n=6), with the mice displaying a grooming pattern more similar to that of the control group. Additionally, MPTP-treated mice demonstrated a reduction in rearing behaviors (standing on hind legs and placing forelegs on the side wall of the cage) (**Fig. 2C**), while coadministration of MPTP and BN52021 significantly improved rearing behaviors (p<0.05, n=6). Furthermore, the duration of immobility, indicative of overall motor impairment, was significantly reduced in the BN52021-treated group compared to the MPTP-treated group (p<0.05, n=6).

MPTP-treated mice exhibited significantly longer traversal times in the pole test compared to the control group (p<0.05, n=6), indicating impaired motor

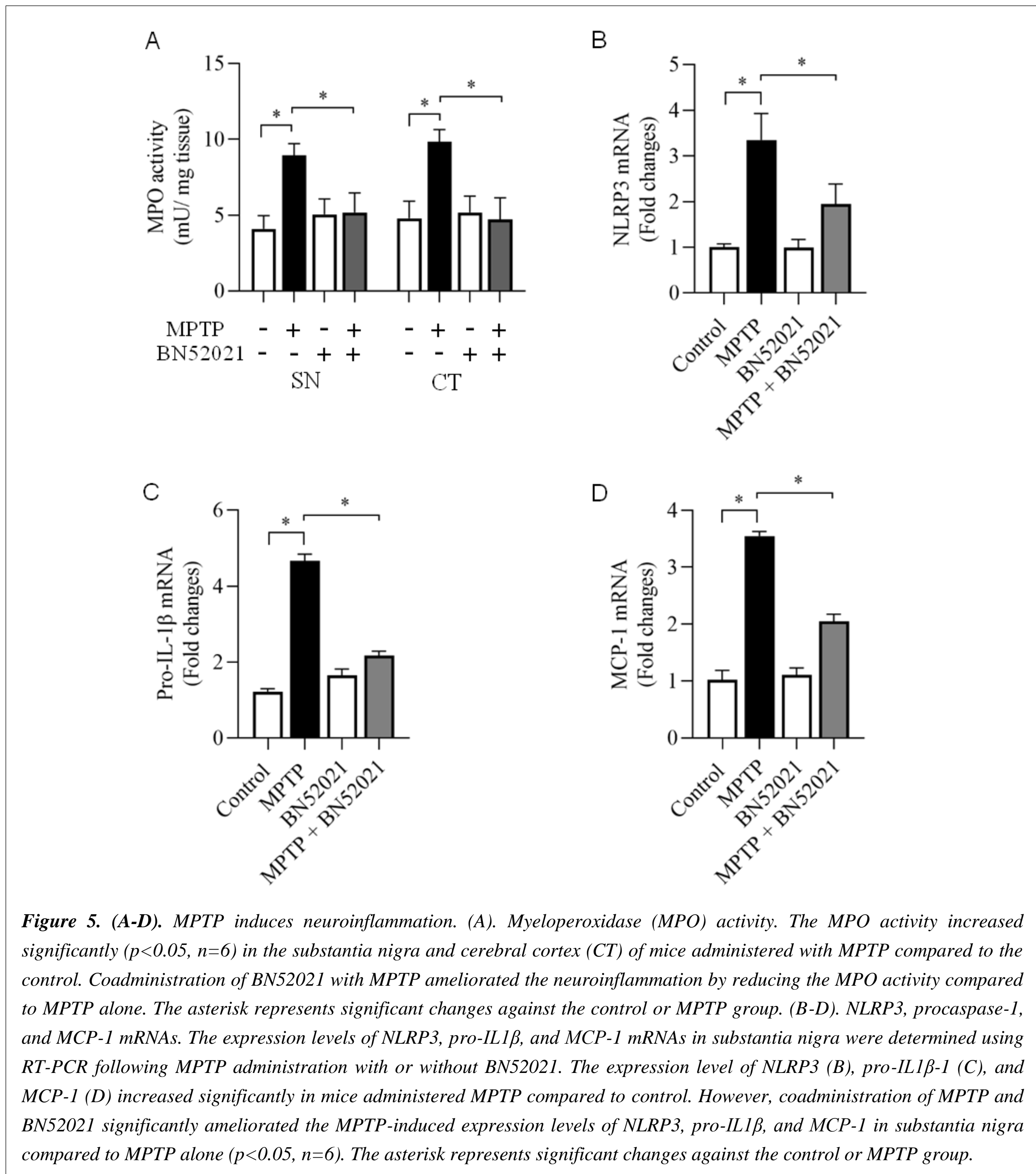

Figure 5. (A-D). MPTP induces neuroinflammation. (A). Myeloperoxidase (MPO) activity. The MPO activity increased significantly (p<0.05, n=6) in the substantia nigra and cerebral cortex (CT) of mice administered with MPTP compared to the control. Coadministration of BN52021 with MPTP ameliorated the neuroinflammation by reducing the MPO activity compared to MPTP alone. The asterisk represents significant changes against the control or MPTP group. (B-D). NLRP3, procaspase-1, and MCP-1 mRNAs. The expression levels of NLRP3, pro-IL1β, and MCP-1 mRNAs in substantia nigra were determined using RT-PCR following MPTP administration with or without BN52021. The expression level of NLRP3 (B), pro-IL1β-1 (C), and MCP-1 (D) increased significantly in mice administered MPTP compared to control. However, coadministration of MPTP and BN52021 significantly ameliorated the MPTP-induced expression levels of NLRP3, pro-IL1β, and MCP-1 in substantia nigra compared to MPTP alone (p<0.05, n=6). The asterisk represents significant changes against the control or MPTP group.

coordination and balance (**Fig. 2D**). However, coadministration of MPTP and BN52021 significantly reduced traversal times compared to the MPTP-treated group ($p<0.05$, n= 6). Notably, mice treated with BN52021 alone showed no significant differences in traversal times compared to the control group (**Fig. 2D**). Additionally, MPTP-treated mice displayed significantly shorter hanging times than the control group ($p<0.05$, n=6), reflecting reduced grip strength and motor endurance (Fig. 2E). In contrast, coadministration of MPTP and BN52021 significantly improved hanging times compared to the MPTP-treated group ($p<0.05$) demonstrating increased grip strength and enhanced motor endurance, as indicated by longer hanging times on the rotating wire (**Fig. 2E**). Importantly, mice treated with BN52021 alone did not exhibit significant differences in hanging times compared to the control group (**Fig. 2E**).

Mitochondrial electron transport complexes

The enzymatic activity of mitochondrial ETC complex I was significantly reduced ($p<0.05$, n=6) in the substantia nigra of MPTP-treated mice compared to the control group (**Fig. 3A**). Coadministration of BN52021 with MPTP significantly enhanced complex I activity compared to both the control and BN52021-alone groups. However, the activities of other mitochondrial ETC, such as complex II (**Fig. 3B**) and complex IV (**Fig. 3C**), remained unchanged in the substantia nigra of mice treated with MPTP, regardless of whether BN52021 was administered. In contrast, mitochondrial complex activities in the cortex were unaffected by MPTP treatment, with or without BN52021 (**Fig. 3A-C**).

Antioxidant enzymes and lipid peroxidation

The enzymatic activities of superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase (**Fig. 4A-D**) decreased significantly in substantia nigra of mice administered with MPTP compared to control ($p<0.05$, n=6). However, coadministration of MPTP and BN52021 significantly ameliorated the MPTP-induced reduction in the antioxidant enzyme activities in substantia nigra compared to MPTP alone ($p<0.05$, n=6). The levels of lipid peroxidation (**Fig.4E**) significantly increased in substantia nigra following MPTP administration compared to control, whereas coadministration of BN52021 with MPTP completely ameliorated the increased lipid peroxidation levels in substantia nigra ($p<0.05$, n=6). In contrast, the activities of antioxidant enzymes in the cortex neither changed nor improved following MPTP administration with or without BN52021 (**Fig. 4A-D**).

Neuroinflammation

MPO activity was significantly elevated ($p<0.05$, n=6) in the substantia nigra and cortex of MPTP-treated mice compared to the control group (**Fig. 5A**). Coadministration of BN52021 with MPTP reduced neuroinflammation by decreasing MPO activity relative to the MPTP-treated group. Additionally, the expression levels of NLRP3 (Fig. 5B), pro-IL-1β (**Fig. 5C**), and MCP-1 (Fig. 5D) were significantly increased in MPTP-treated mice compared to control. However, coadministration of BN52021 with MPTP significantly attenuated the MPTP-induced increases in NLRP3, pro-IL-1β, and MCP-1 expression in the substantia nigra ($p < 0.05$, n = 6) compared to MPTP alone.

Discussion

The present study demonstrates the effects of Ginkgolide B (BN52021) in ameliorating Parkinson's-like conditions in murine models. When administered to mice, MPTP selectively damages dopaminergic neurons in the substantia nigra, leading to motor impairments similar to those observed in PD patients. The progressive loss of dopaminergic neurons in the substantia nigra results in a reduction of dopamine levels in the striatum (Dauer et al., 2003), motor impairments, such as bradykinesia (slowness of movement), rigidity, and tremors, which are the hallmark characteristics of PD. The MPTP-induced PD mouse model has been instrumental in understanding the pathophysiology of PD (Jackson-Lewis et al., 2007), testing potential therapeutic interventions, and evaluating the efficacy of novel drugs.

The results demonstrate that BN52021 effectively mitigates MPTP-induced motor deficits and behavioral impairments in mice, providing potential therapeutic insights for PD management. The observed reduction in gridline crossings, grooming behaviors, rearing activity, and performance in both the pole and hanging tests highlight the severe motor impairments and behavioral changes induced by MPTP, a well-established model for PD-like symptoms. However, coadministration of BN52021 significantly improved motor coordination, balance, grip strength, and endurance, as evidenced by improvements in the open field, pole, and hanging tests. In

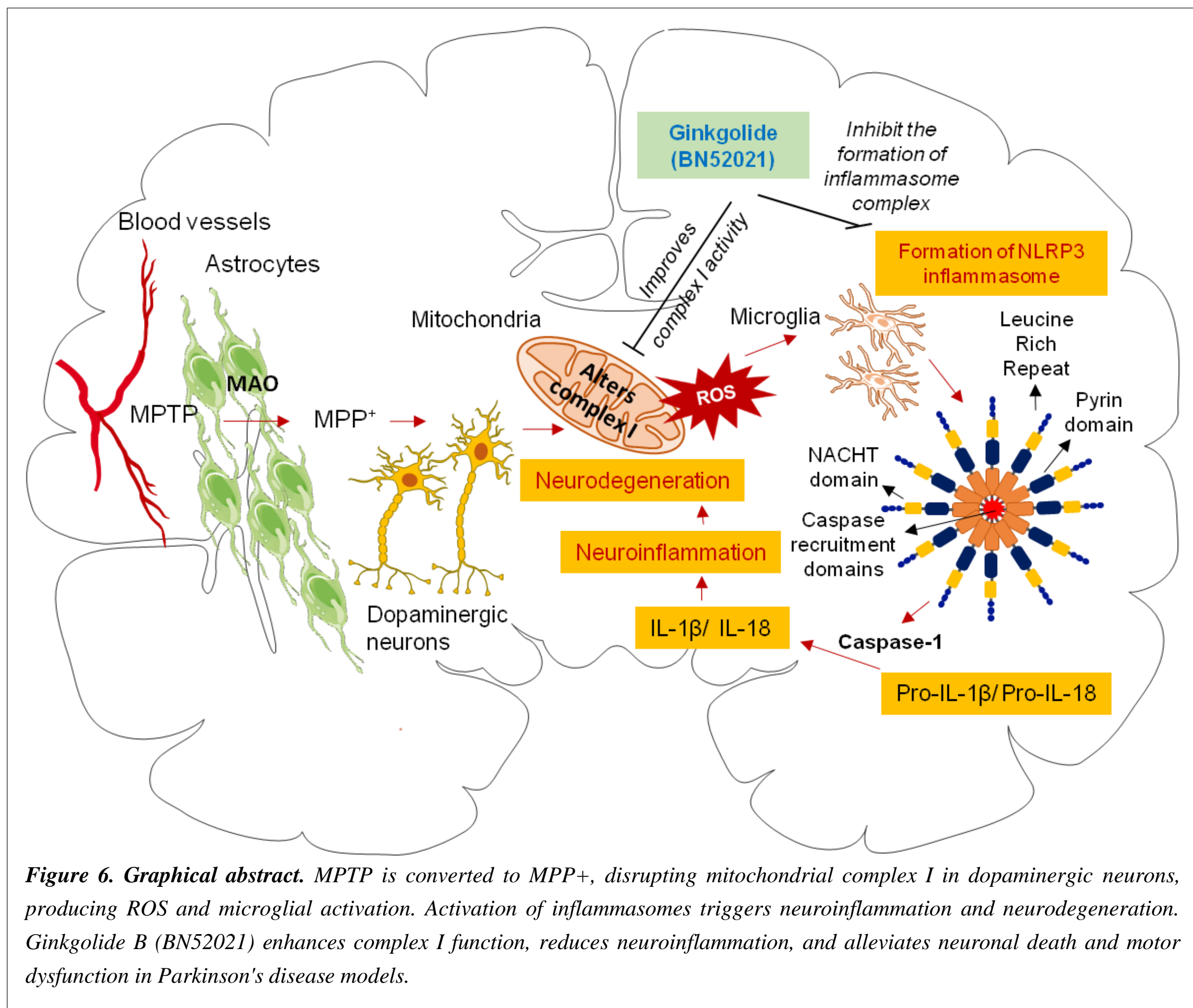

Figure 6. Graphical abstract. *MPTP is converted to MPP+, disrupting mitochondrial complex I in dopaminergic neurons, producing ROS and microglial activation. Activation of inflammasomes triggers neuroinflammation and neurodegeneration. Ginkgolide B (BN52021) enhances complex I function, reduces neuroinflammation, and alleviates neuronal death and motor dysfunction in Parkinson's disease models.*

the present study, MPTP-treated mice showed overall reduced locomotor activities compared to the control group, indicating impaired mobility, which agrees with previous studies (Tillerson et al., 2002). However, coadministration of MPTP and BN52021 significantly improved locomotor activities, including increased mobility and reduced abnormal movement patterns such as hind leg dragging.

Furthermore, mitochondrial dysfunction is a critical factor in the pathogenesis of PD. In the present study, MPTP significantly decreased mitochondrial complex I activity, specifically in the substantia nigra, while complexes II and IV remained unaffected in both the substantia nigra and cortex. This selective impairment of complex I is consistent with previous reports showing that MPTP primarily disrupts the ETC by inhibiting complex I, reducing ATP production, and increasing oxidative stress (Ramsay et al., 1986; Suzuki et al., 1990; Mizuno et al., 1990). Interestingly, treatment with BN52021 restored complex I activity, indicating its potential to improve mitochondrial bioenergetics in the substantia nigra, a critical region vulnerable to PD-related neurodegeneration. The lack of changes in complex II and IV activity across both the substantia nigra and cortex suggests that the primary target for MPTP is complex I, further underscoring the importance of this complex in PD pathology. The protective effect of BN52021 on complex I may involve the modulation of oxidative stress and mitochondrial integrity, as complex I dysfunction is tightly linked to increased ROS. MPTP exposure led to a marked reduction in the activities of antioxidant enzymes, such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase, specifically in the substantia nigra. In contrast, enzyme levels in the cortex remained unchanged, suggesting that the substantia nigra, which is rich in dopaminergic neurons, is particularly susceptible to oxidative stress caused by MPTP. BN52021 administration significantly restored antioxidant enzyme levels in the

substantia nigra, highlighting its role in counteracting oxidative stress. These findings align with previous studies indicating that oxidative stress is a major contributor to dopaminergic neuronal death in PD (Suzuki et al., 1990) and suggest that BN52021 enhances the brain's antioxidant defenses, thus providing neuroprotection.

Neuroinflammation is increasingly recognized as a pivotal mechanism in the progression of PD. In this study, MPTP significantly elevated inflammatory markers, including the activities of myeloperoxidase, in both the substantia nigra and cortex. Additionally, the gene expression of key inflammasome and proinflammatory cytokines, NLRP3, pro-IL-1β, and MCP-1 was upregulated in the substantia nigra following MPTP treatment, suggesting that activation of the NLRP3 inflammasome pathway, which has been implicated in the neuroinflammatory processes contributing to PD pathogenesis (Pike et al., 2022; Sivagurunathan et al., 2024; Brahadeeswaran et al., 2022). Notably, BN52021 attenuated the MPTP-induced elevation of MPO in both brain regions and downregulated the expression of NLRP3, pro-IL-1β, and MCP-1 in the substantia nigra. These results suggest that BN52021 exerts its neuroprotective effects by modulating the inflammatory response. The suppression of NLRP3 inflammasome activation may be particularly critical, as this pathway is associated with releasing proinflammatory cytokines that exacerbate further oxidative stress-induced neuronal damage. By inhibiting these inflammatory processes, BN52021 reduces neuroinflammation, which could slow the progression of neurodegeneration in PD.

In cocnlusion, the present study concludes that BN52021 mitigates MPTP-induced neurotoxicity by improving mitochondrial complex I activity, enhancing antioxidant defenses, and attenuating neuroinflammation, particularly in the substantia nigra (Fig. 6). The neuroprotective effects of BN52021 make it a promising candidate for further investigation as a potential therapeutic agent for PD. Future studies should explore the underlying mechanisms of actions of BN52021, including its effects on mitochondrial dynamics and its long-term impact on the survival of dopaminergic neurons and motor functions.

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Declaration of competing interest
None to declare

Disclosure of ethical statements
Approval of the Research Protocol: N/A
Informed Consent: N/A

Registry and Registration No. of the Study: The present animal studies were approved by the Institutional Animal Ethics Committee, Rajah Muthiah Medical College, Annamalai University (Approval ID: AU-IAEC/PR/1283/10/20).

Animal Studies: All animal studies were carried out following the national and international guidelines and the relevant national laws on the protection of animals.

Patient Consent for Publication: N/A

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