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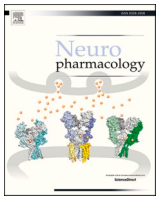

Dopaminergic neuroprotective effects of inosine in MPTP-induced parkinsonian mice via brain-derived neurotrophic factor upregulation

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ABSTRACT

Parkinson's disease (PD) is the second most common neurodegenerative disease. However, no curative or modifying therapy is known. Inosine is a purine nucleoside that increases brain-derived neurotrophic factor (BDNF) expression in the brain through adenosine receptors. Herein, we investigated the neuroprotective effects of inosine and elucidated the mechanisms underlying its pharmacological action. Inosine rescued SH-SY5Y neuroblastoma cells from MPP^+ injury in a dose-dependent manner. Inosine protection correlated with BDNF expression and the activation of its downstream signaling cascade, as the TrkB receptor inhibitor, K252a and siRNA against the BDNF gene remarkably reduced the protective effects of inosine. Blocking the A_1 or A_{2A} adenosine receptors diminished BDNF induction and the rescuing effect of inosine, indicating a critical role of adenosine A_1 and A_{2A} receptors in inosine-related BDNF elevation. We assessed whether the compound could protect dopaminergic neurons from MPTP-induced neuronal injury. Beam-walking and challenge beam tests revealed that inosine pretreatment for 3 weeks reduced the MPTP-induced motor function impairment. Inosine ameliorated dopaminergic neuronal loss and MPTP-mediated astrocytic and microglial activation in the substantia nigra and striatum. Inosine ameliorated the depletion of striatal dopamine and its metabolite following MPTP injection. BDNF upregulation and the activation of its downstream signaling pathway seemingly correlate with the neuroprotective effects of inosine. To our knowledge, this is the first study to demonstrate the neuroprotective effects of inosine against MPTP neurotoxicity via BDNF upregulation. These findings highlight the therapeutic potential of inosine in dopaminergic neurodegeneration in PD brains.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the preferential loss of dopaminergic neurons in the substantia nigra and a consequent deficiency in striatal dopamine. Dopaminergic neurodegeneration causes motor symptoms including resting tremor, bradykinesia, rigidity, and postural instability (Gökçal et al., 2017). In addition to motor symptoms, non-motor symptoms, such as sleep disturbance, anxiety, depression, cognitive impairment, hyposmia, and constipation, occur in patients (Schapira et al., 2017; Marinus et al., 2018; Antony et al., 2013). Although there has been intensive research on the pathogenesis of PD, the precise molecular mechanism remains elusive; consequently, no known cure exists for this disease.

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family and has been known to play a key role in neuronal survival (Kowiański et al., 2018), migration (Ortiz-López et al., 2017), and synaptogenesis (Miranda et al., 2019; Wang et al., 2022). Neuropeptides protect neurons from injury by suppressing the apoptotic cascade. BDNF binds to its receptor (TrkB) and induces receptor dimerization, which activates the Akt/PI3K and CREB pathways. These pathways are involved in neuronal survival, growth, and synaptic plasticity (Pradhan et al., 2019). Multiple studies have demonstrated

Abbreviations: PD, Parkinson's disease; BDNF, Brain-derived neurotrophic factor; Trk-B, Tropomyosin receptor kinase B; CREB, cyclic AMP response element-binding protein; Akt, Protein kinase B; PI3K, Phosphoinositide 3-kinase; MPP^+, 1-Methyl-4-PhenylPyridinium; MPTP, 1-Methyl- 4-Phenyl-1,2,3,6-Tetrahydropyridine; CCK-8, Cell counting kit-8; DOPAC, 3,4-Dihydroxyphenylacetic acid; TH, Tyrosine hydroxylase; EDTA, Ethylenediaminetetraacetic acid; GFAP, Glial fibrillary acidic protein; Iba1, Ionized calcium-binding adapter molecule 1; PCA, Perchloric acid; UPDRS, Unified Parkinson's disease rating scale.

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potent neuroprotective activity of neuropeptides in various neurodegenerative diseases (Chen et al., 2013b; Zuccato and Cattaneo, 2009). Furthermore, reduced BDNF levels in the substantia nigra are apparently associated with the death of nigral dopaminergic neurons in PD (Howells et al., 2000a; Nagahara and Tuszynski, 2011). Despite the remarkable protective property of BDNF, its therapeutic application is limited because the peptide cannot cross the blood-brain barrier. The enhancement of BDNF expression in the brain by employing viral vectors and the transplantation of gene-modified glial cells is of interest, but entails some hurdles. Thus, BDNF upregulation in the brain using specific drugs is an optimal strategy for treating neurodegenerative diseases (Khan et al., 2018; Azman and Zakaria, 2022). Several compounds can induce BDNF, which is related to the amelioration of neurological symptoms, such as depression, parkinsonism, and cognitive impairment (Srivastav et al., 2019; Bhurtel et al., 2018; Shadfar et al., 2018). TrkB activators, such as 7,8-dihydroxyflavone, also show beneficial effects in neurological disorders (Zuo et al., 2021; Nie et al., 2019). In this context, inosine is an endogenous compound that upregulates BDNF levels in the brain. Moreover, inosine exhibits anti-depression (Muto et al., 2014), anti-Alzheimer's disease (Teixeira et al., 2022), and anti-Huntington's disease (El-Shamarka et al., 2022)effects via the BDNF signaling pathway, suggesting that inosine is a promising candidate for the treatment of neurological diseases such as PD.

Inosine can bind to one of four subtypes of adenosine receptors including A_1, A_{2A}, A_{2B}, and A3 and activate them (Muto et al., 2014). Calcium influx is blocked when the A_1 receptor is engaged, reducing excitatory neurotransmitter release at both the presynaptic and postsynaptic levels. Over the years, several efforts have been made to develop A_1 receptor agonists that protect against cerebral ischemia and brain injury (Abbracchio and Cattabeni, 1999). A recent study reported that the administration of inosine increased BDNF transcription in the hippocampus, which was partially blocked by adenosine A_1 and A_{2A} receptor antagonists, suggesting that inosine upregulates BDNF expression in the brain through A_1 and A_{2A} receptors (Muto et al., 2014).

In this study, we evaluated the neuroprotective effects of inosine *in vitro* and *in vivo*. We demonstrated the dopaminergic neuroprotective effects of inosine, which may be attributable to BDNF upregulation in the brain. To our knowledge, this is the first report that shows the neuroprotective effects of inosine in a PD model via BDNF induction.

2. Materials and methods

2.1. SH-SY5Y cell culture

SH-SY5Y human neuroblastoma cells were provided by Professor Gil-Saeng Jeong (College of Pharmacy, Keimyung University). The cells were cultured at 37 °C in a 5% CO_2 humidified atmosphere in Dulbecco's modified Eagle's medium (Product #SH30243.01, HyClone Laboratories), supplemented with 10% fetal bovine serum (Product #SH30084.03, HyClone Laboratories) and 1% penicillin/streptomycin (Product #SV30010, HyClone Laboratories). The cells were cultured until they attained 80–90% confluency, after which they were trypsinized with 0.25% trypsin (Product #SH30042.01, HyClone Laboratories) and subcultured or plated for treatment of the cells with appropriate cell densities. The cell culture medium was replaced every 2–3 days.

2.2. Treatment with inosine, MPP^+, and inhibitors of TrkB, Akt, ERK and adenosine receptor

The cells were treated with inosine (TCI Chemicals, Japan) in concentrations ranging of 1–200 μM 2 h before MPP^+ iodide (Cayman Chemicals, USA) and incubated for 24 h. 100 nM of K252a (SF2370, Alomone labs), a TrkB receptor inhibitor was treated with the cells 1 h before inosine treatment. 5 μM of 10-DEBC (Akt inhibitor, Product #2558, Tocris), 5 μM of U0126 (ERK1/2 inhibitor, Product #9903, Cell Signaling Technology), 100 μM of A_1 receptor antagonist, DPCPX (0439, Tocris, United Kingdom), A_{2A} receptor antagonist, SCH58261 (2270, Tocris, United Kingdom), or A_{2B} receptor antagonist, PSB 1115 (2009, Tocris, United Kingdom) was added to the cell culture medium, 1 h before inosine.

2.3. Cell viability assay

Cell viability was determined using the cell counting kit-8 (CCK-8) assay (Product #CK04, Dojindo Laboratories, Japan). SH-SY5Y cells (1×10^4/well) were plated in a 96-well plate, and 10 μL of CCK-8 reagent was added to each well. Absorbance was measured at 450 nm using a microplate reader (Multiskan GO, Thermo Scientific, USA), 2 h after incubation with the reagent. Cell viability was expressed as a percentage of the control group.

2.4. Western blot analysis

Cells and brain tissues were transferred into tubes containing pre-chilled RIPA lysis buffer and 1% protease inhibitor cocktail and homogenized using a sonicator. The supernatants were collected in tubes after homogenization and centrifugation at 13,000 rpm for 20 min at 4 °C. A BCA protein quantitation test kit (ThermoFisher Scientific, USA) was used to quantify protein concentrations. Before transfer onto PVDF membranes (Millipore Corporation, Temecula, CA, USA), equal amounts of proteins were loaded and resolved in a 12% SDS-polyacrylamide gel. The membrane was blocked in 5% skim milk prepared in Tris-buffered saline containing 1% Tween20 for 1 h and then incubated with primary antibodies against BDNF (1:1250; Santa Cruz Biotechnology), p-Akt (1:5000; Cell Signaling Technology), Akt (1:5000; Cell Signaling Technology), p-CREB (1:2000; Invitrogen), CREB (1:2000; Invitrogen), p-ERK1/2 (1:2000; Cell Signaling Technology), ERK1/2 (1:2000; Cell Signaling Technology), β-actin (1:5000; AB frontiers), or GAPDH (1:5000; AB frontiers) at 4 °C overnight. The membrane was then treated for 1 h at 23 °C with an HRP-labeled secondary antibody. The membrane was exposed to enhanced chemiluminescence reagents (ThermoFisher Scientific) and the resultant chemiluminescence was assessed using an analyzer (Fusion Solo; Vilberlourmat). The density of each blot was assessed using GelQuant.Net software.

2.5. siRNA transfection with the SH-SY5Y cells

Using HiPerfect transfection reagent (#301705, Qiagen, Maryland, USA), SH-SY5Y cells were transfected with siRNA targeting the BDNF gene (Sense: 5′-GUGCUUUAAGUGCCUACAU-3′, Anti-sense: 3′-AUGUAGGCACUUAAAGCAC-5′) (Bioneer, Daejeon, South Korea) or non-specific scrambled siRNA (Bioneer, Daejeon, South Korea). The cells were plated in 35 mm culture dishes at a density of 2×10^5 cells/dish and incubated overnight. The culture medium containing the transfection mixture was introduced into the cells and incubated at 23 °C for 6 h. The cells were treated with 0.1% FBS and cultured for 24 h. The cells were rinsed in 1 × PBS before treatment with the compounds, as indicated in the figure legends. Western blotting was used to assess the efficacy of gene silencing.

2.6. Reverse transcriptase-polymerase chain reaction (RT-PCR)

To analyze the gene expression, the total RNA was isolated from the treated cells using Trizol reagent (Product #97306, Qiagen, Valencia, CA). Thermo Scientific Drop Plate, photometric nucleic acid quantification (Thermo Scientific Multiskan GO microplate and cuvette spectrophotometer) was used for determining the quantity and quality of RNA. GoScript Reverse Transcription System (Promega, Madison, WI) was used to reverse-transcribe 1 μg of RNA into cDNA. On a thermal cycler (Thermo Fisher Scientific), cDNA amplification for various genes was carried out using the Taq polymerase master mix (Bioneer, South

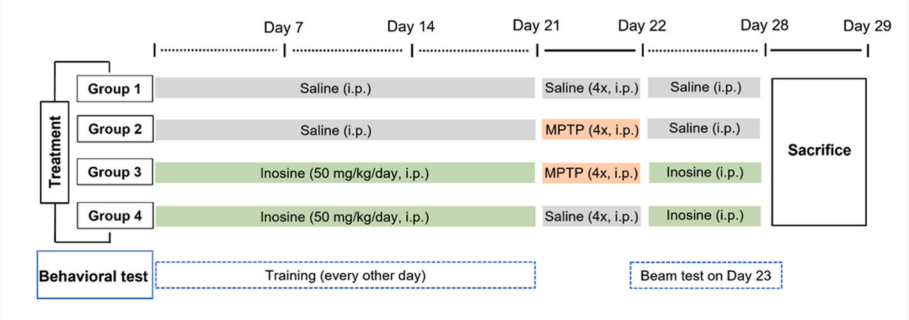

Fig. 1. Schematic diagram of experimental design of the animal study.

Korea) and forward/reverse primers from Bioneer. The resultant DNA was visualized in 2% agarose gel. GAPDH bands were used as a loading control for the quantitative standardization of mRNA of each samples. The primer sequence for BDNF and GAPDH are: BDNF Forward 5′- CGT GTT CGA AAG TGT CAG CC-3′, Reverse 5′- CTT GGA CAG AGC CAA CGG AT-3′ and GAPDH Forward 5′-ACC ACA GTC CAT GCC ATC AC-3′, Reverse 5′- TCC ACC ACC CTG TTG CTG TA-3'.

2.7. Animals and drug treatment

Adult C57BL/6 male mice (10 weeks old, 20–25 g, Orient Bio, Seoul, Korea) were housed in microisolator cages with a 12-h light/dark cycle, and food and water were provided *ad lib*. Animal experiments were performed in accordance with the protocol approved by the Instructional Animal Care and Use Committee of Yeungnam University (approval number:2020-012). After one week of acclimation, the animals were divided into four groups (5 mice/group). Group 1 received normal saline for 21 days and intraperitoneal saline injections; Group 2 received an injection of inosine (50 mg/kg/day) for 21 days and then saline; Group 3 received a saline injection for 21 days and MPTP (15 mg/kg × 4 at 1.5-h intervals); Group 4 received inosine for 21 days followed by MPTP.

2.8. Behavioral tests

Behavioral tests were performed after MPTP injection to measure the motor deficits caused by MPTP treatment in animals. The following tests were performed:

2.8.1. Beam traversal test

In this procedure, a 100-cm long wooden beam with four equal parts ranging in width from 3.5 cm to 0.5 cm was positioned above the floor in such a way that the thinnest beam led to the home cage. Each mouse was housed at the widest point of the beam and trained to wander along with it into its home cage. Before the day of the experiment, each group's animals were taught three times for a total of five days. After being used by each animal, the beam was properly cleaned. All beam tests were recorded with a video camera, and the time taken by each mouse to reach the home cage was recorded with a stopwatch (from the wider to the narrower end). The data were presented as the average of three trials per animal.

2.8.2. Challenge beam traversal test

A mesh grid (1 cm squares) with a width equivalent to the size of the beam was placed on the beam surface. Animals were allowed to walk over the mesh, and the time taken to reach the home cage was measured using a stopwatch. Data were presented as the mean of three trials per animal.

2.9. Analysis of neurochemicals

High-performance liquid chromatography (HPLC) (1260 Infinity, Agilent Technologies) with an electrochemical detector (Coulochem III; ThermoFisher Scientific) was used to examine the striatal concentrations of monoamines. The tissues of the striatum were weighed and homogenized in ice-cold 0.1 N perchloric acid (PCA). The homogenate was centrifuged for 20 min at 13000 rpm and 4 °C, and the supernatant was collected. Standard solutions of dopamine and DOPAC were prepared using serial dilutions. With a flow rate of 0.6 ml/min, 15 μl of each supernatant or standard were injected into the mobile phase (75 mM sodium phosphate monobasic, 1.7 mM 1-octane sulfonic acid, 100 μl/L Triethylamine, 25 μM EDTA, 15% acetonitrile).

2.10. Immunohistochemistry

The right hemisphere of the brain was fixed in 4% paraformaldehyde, and coronal slices (30 μM) were prepared using a freezing sliding microtome (Microm HM 450; ThermoFisher Scientific) and preserved in a cryoprotectant solution. Immunostaining was performed on free-floating brain slices. Endogenous peroxidase activity was suppressed by incubating the brain sections with 3% hydrogen peroxide. The slices were then incubated with rabbit polyclonal primary antibodies against TH (1:3000; ThermoFisher Scientific), GFAP (1:3000; ThermoFisher Scientific) or Iba1 (1:2000; Wako, Japan) in KPBS containing 0.4% Triton X-100 (ThermoFisher Scientific) overnight at 4 °C. The sections were then treated with biotinylated secondary antibody for 2 h (1:1000, Vector Laboratories Inc.). The resultant cells were mounted on glass slides, coverslipped, and observed under a light microscope (BX41 TF, Olympus, Japan). The number of TH-positive cells was counted in the sections covering the whole substantia nigra using a blind count.

The extent of astroglial activation and microglial activation in the striatum and substantia nigra were assessed as described by (Jung et al., 2017). First, substantia nigra and striatum microscopic digital pictures were captured using an Olympus microscope equipment equipped with an AcquCAM pro/G3 camera (Germany). Utilizing the ImageJ program (available at http://rsbweb.nih.gov/ij/), GFAP and Iba1 immunoreactivity were assessed. To assess the number of pixels above the threshold, the threshold value was set to 200. All numbers were represented as a percentage of all the pixels in the microscope field.

2.11. Statistical analysis

All values represented the mean ± S.E.M. of independent experimental results. Ordinary one-way ANOVA was used for the statistical analyses, followed by the Tukey's multiple comparison test using GraphPad Prism 9.0 (San Diego, CA). Statistical significance was defined as p-values <0.05.

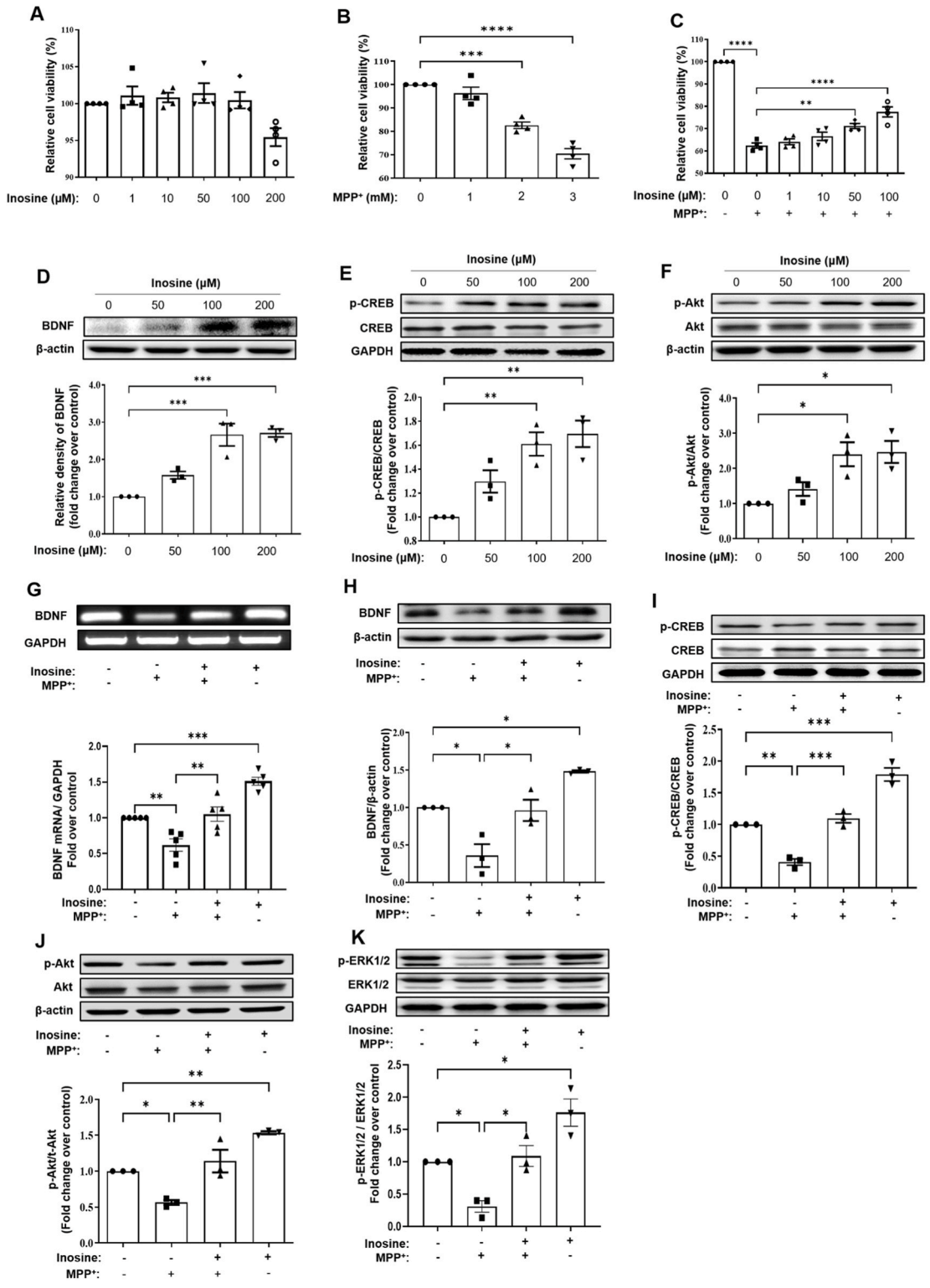

Fig. 2. Effects of inosine on MPP $^{+}$ -induced loss of SH-SY5Y neuroblastoma cells and expression of BDNF and its downstream molecules.
The SH-SY5Y cells were exposed to MPP^{+} in the presence or absence of inosine, and then the cell viability was assessed using CCK-8 assay. In addition, Western blots were performed to measure levels of BDNF, CREB and Akt. (A) Effects of inosine ranging from 1 to 200 μM on viability of SH-SY5Y cells. (B) Loss of SH-SY5Y cell caused by exposure to MPP^{+} in a dose-dependent manner. (C) Rescuing effect of inosine from MPP^{+} injury in a dose-dependent manner. (D–F) Upregulation of BDNF, phospho-CREB and phospho-Akt in the SH-SY5Y cells after treatment with inosine. (G–K) Downregulation of BDNF, phospho-CREB, phospho-Akt and phospho-ERK in the SH-SY5Y cells after exposure to MPP^{+}, which was counteracted by treatment with inosine (100 μM). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. All values represent mean ± S.E.M. (n = 4).

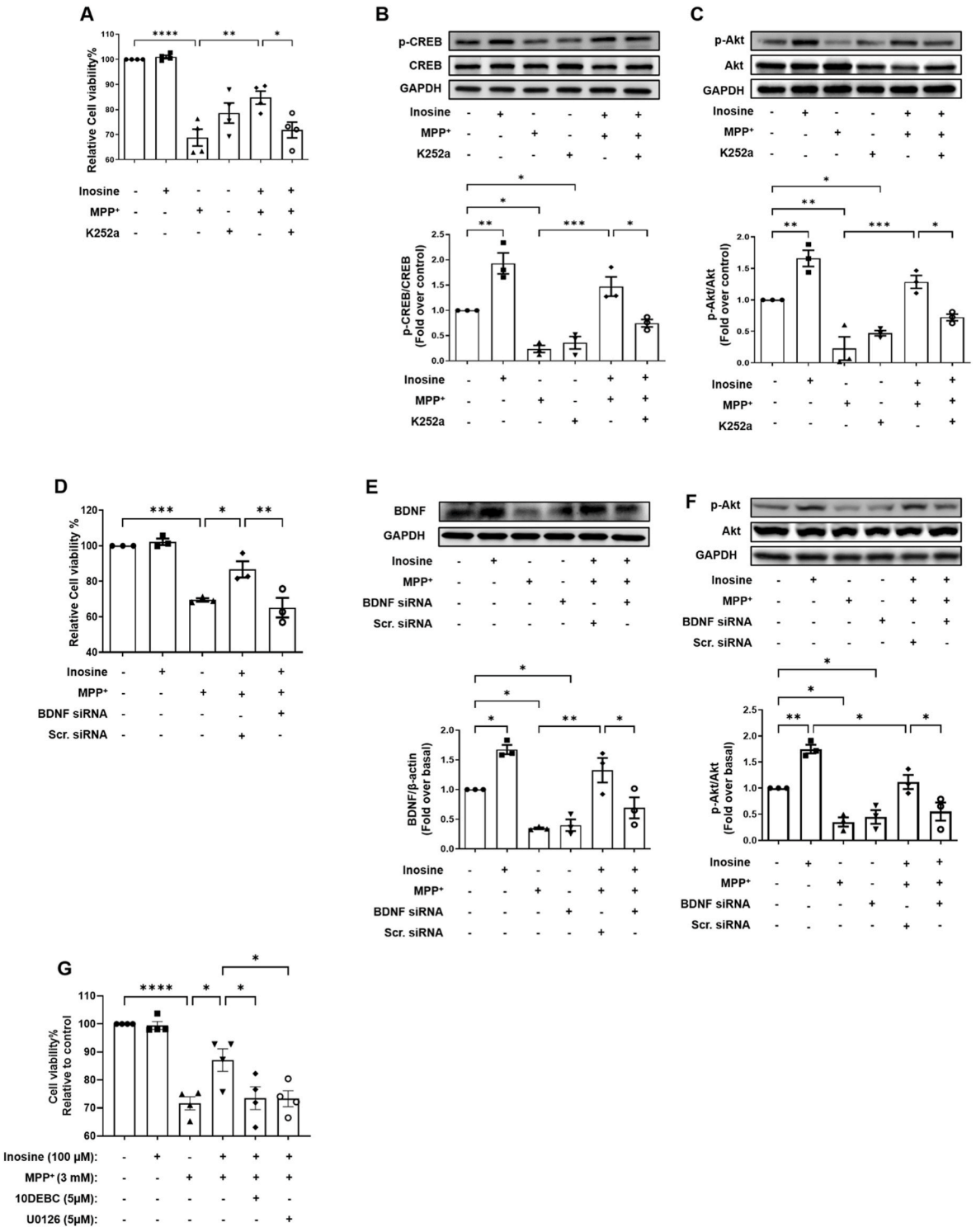

Fig. 3. Association of BDNF-TrkB pathway with inosine's protective activity against the MPP^+-induced cytotoxicity.
Effects of K252a, an antagonist of TrkB receptor on inosine's cytoprotection against MPP^+-induced cell death and upregulation of phospho-CREB and phospho-Akt were evaluated in the SH-SY5Y cells treated with or without MPP^+. In addition, effects of siRNA against BDNF gene on inosine's protective activity against MPP^+ toxicity and upregulation of BDNF and phospho-Akt were assessed in the presence or absence of MPP^+. Further, we tested if inhibition of Akt or ERK reversed protective action of inosine. (A) Effects of K252a on rescuing action of inosine from MPP^+-induced cell injury. (B–C) Effects of K252a on upregulation of phospho-CREB and phospho-Akt by inosine. (D) Effects of siRNA against BDNF gene on rescuing action of inosine from MPP^+-induced cell injury. (E–F) Effects of siRNA against BDNF gene on upregulation of BDNF and phospho-Akt by inosine. (G) Counteraction of protective action of inosine upon addition of inhibitor of Akt (10-DEBC) or ERK (U0126). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. All values represent mean ± S.E.M. (n = 3).

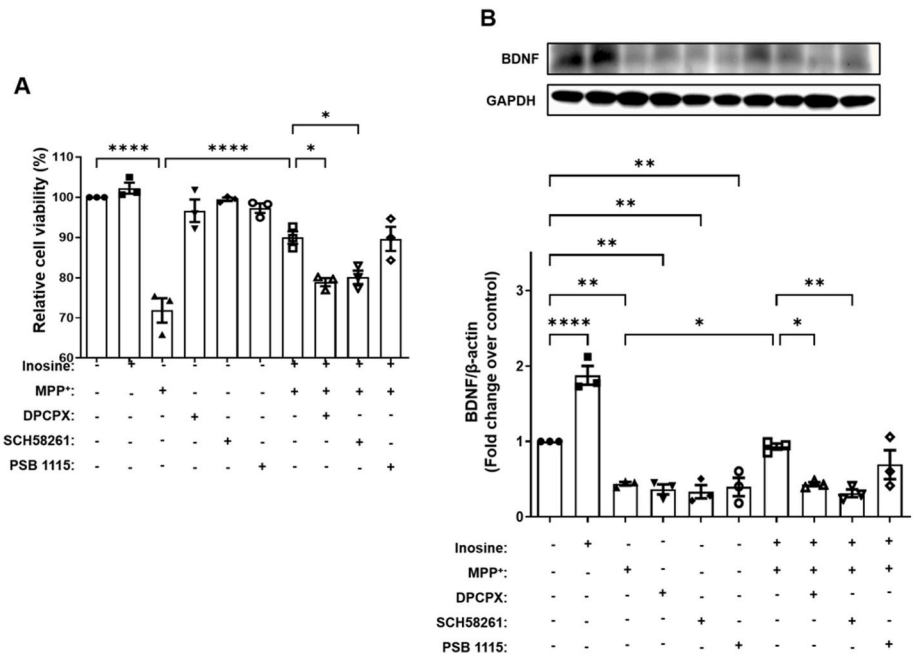

Fig. 4. Involvement of A_1 and A_{2A} adenosine receptors in BDNF upregulation by inosine.
The SH-SY5Y cells were exposed to DPCPX (A_1 antagonist), SCH58261 (A_{2A} antagonist) or PSB1115 (A_{2B} antagonist) in the presence or absence of inosine or MPP^+. And then cell viability test was performed using CCK-8 assay, and Western blot analyses were conducted to measure the level of BDNF in the SH-SY5Y cell. (A) Effects of antagonism against A_1, A_{2A} or A_{2B} receptor on inosine's protection from MPP^+-induced cell loss. (B) Effects of antagonism against A_1, A_{2A} or A_{2B} receptor on inosine's BDNF-restoring action. $^*p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$. All values represent mean ± S.E.M. (n = 3).

3. Results

3.1. Inosine protects SH-SY5Y cells from MPP^+-induced cytotoxicity

First, we investigated whether inosine is toxic to neuroblastoma cells in the concentration range of 1–200 μM. Inosine did not seem toxic to the cells in this concentration range, as shown in Fig. 1A. Next, we tested whether inosine could rescue SH-SY5Y cells from MPP^+-induced injury. MPP^+ (3 mM) seemed appropriate for testing the cytoprotective effect of inosine, as approximately 30% of the cells were killed at this concentration; therefore, we selected 3 mM MPP^+ for further studies ($F_{(3, 12)} = 5$, $p < 0.0001$) ((Fig. 2B). The MTS assay revealed that MPP^+ caused significant loss of the cells ($p < 0.0001$), and 50 and 100 μM inosine significantly protected against MPP^+ toxicity ($p = 0.058$ and $p < 0.0001$) ($F_{(5, 18)} = 91.80$ $p < 0.0001$) (see Fig. 2C). According to a previous study, inosine upregulates BDNF expression in the brain (Gonçalves et al., 2017). Thus, we used Western blot analysis to determine whether inosine could increase neurotrophic factor expression and activate downstream signaling pathways, including CREB and Akt. SH-SY5Y cells were treated with three different concentrations of inosine (50, 100, and 200 μM) and incubated for 24 h. Inosine significantly increased BDNF levels in a dose-dependent manner ($F_{(3, 8)} = 25.44$, $p = 0.0002$), (100 μM, $p = 0.0005$ and 200 μM, $p = 0.0004$) (Fig. 2D). Furthermore, the phosphorylation of CREB and Akt was elevated in a dose-dependent manner ($F_{(3,8)} = 13.16$, $p = 0.0018$) (100 μM, $p = 0.005$ and 200 μM, $p = 0.0022$ (Fig. 2E) and ($F_{(3,8)} = 8.4$, $p = 0.0075$)(100 μM, $p = 0.018$ and 200 μM, $p = 0.014$ (Fig. 2F). Inosine (100 μM) was selected for further studies based on the results of cell viability and BDNF induction by inosine.

We assessed whether BDNF induction by insone was related to its protective effects. SH-SY5Y cells were pretreated with 100 μM inosine for 2 h followed by MPP^+ for 24 h, and the levels of BDNF, Akt, and CREB were evaluated. The result of RT-PCR confirmed that MPP^+ remarkably decreased the mRNA level of BDNF ($p = 0.0092$) while inosine pretreatment restored the BDNF mRNA expression ($p = 0.0033$) ($F_{(3,16)} = 2$, $p < 0.0001$) (Fig. 2G). Furthermore, Western blot results revealed that MPP^+ significantly decreased BDNF ($p = 0.0106$) p-CREB ($p = 0.0011$), and p-Akt ($p = 0.0219$), p-ERK1/2 ($p = 0.022$) in the cells, and inosine restored their levels, BDNF ($p = 0.015$), p-CREB ($p = 0.0004$), p-Akt ($p = 0.0045$) ($F_{(3,8)} = 19.37$, $p = 0.0005$), p-ERK1/2 ($p = 0.0006$) ($F_{(3,8)} = 18$, $p = 0.0006$) (Fig. 2H–K), collectively implying that the neuroprotective effects of inosine relies on BDNF upregulation.

3.2. Neuroprotective effects of inosine against MPP^+ toxicity are mediated by BDNF/TrkB/Akt/ERK1/2 signaling pathway

We hypothesized that the neuroprotective effects of inosine rely on BDNF upregulation. To test this hypothesis, we treated SH-SY5Y cells with the TrkB receptor-specific inhibitor, K252a (Tapley et al., 1992) for 1 h and then inosine and MPP^+, sequentially. The results showed that inosine attenuated the loss of cells mediated by MPP^+ ($p = 0.0287$), which was blocked by addition of K252a ($p = 0.0072$) ($F_{(4,10)} = 25.97$, $p < 0.0001$) (Fig. 3A). Furthermore, inosine restored the levels of p-CREB ($p = 0.0003$), and p-Akt ($p = 0.0001$), which were reduced by MPP^+ ($p = 0.0152$, $p = 0.0024$). Upon treatment with K252a, the restorative effects of inosine clearly diminished ($p = 0.0213$) ($F_{(5,12)} = 1$, $p < 0.0001$) (Fig. 3B–D).

To confirm the role of BDNF in the neuroprotective effects of inosine, SH-SY5Y cells were transfected with 100 nM BDNF siRNA for 24 h. The siRNA concentration was selected as the optimal concentration by evaluating the transfection efficiency (data not shown). The cell viability tests revealed that the protective effects of inosine were suppressed by transfection with siRNA against BDNF ($p = 0.0072$) ($F_{(4,10)} = 2$, $p < 0.0001$) (Fig. 3E). Western blot analysis further revealed that BDNF silencing blocked the restorative effects of inosine on BDNF ($p = 0.0304$) ($F_{(5,12)} = 1$, $p < 0.0001$)and p-Akt levels ($p = 0.0423$) ($F_{(5,12)} = 2$, $p < 0.0001$) (Fig. 3F and G).

Also, to confirm the additional role of Akt and ERK1/2 in inosine-induced neuroprotection, SH-SY5Y cells were pretreated with 5 μM of 10-DEBC (Akt inhibitor), or 5 μM of U0126 (MEK1/2 inhibitor; also ERK1/2 inhibitor) for 1 h, followed by 2 h treatment of inosine and MPP^+ for 24 h. The result of cell viability displayed that neuroprotective effects of inosine were significantly reversed by 10-DEBC ($p = 0.032$) and U0126 ($p = 0.029$) ($F_{(5,18)} = 21.96$, $p < 0.0001$) (Fig. 3G).

3.3. A_1 and A_{2A} receptors are involved in the inosine-mediated BDNF upregulation and cytoprotection

To explore the role of adenosine receptors in the cytoprotection of inosine against MPP^+ and in the induction of BDNF by inosine, SH-SY5Y

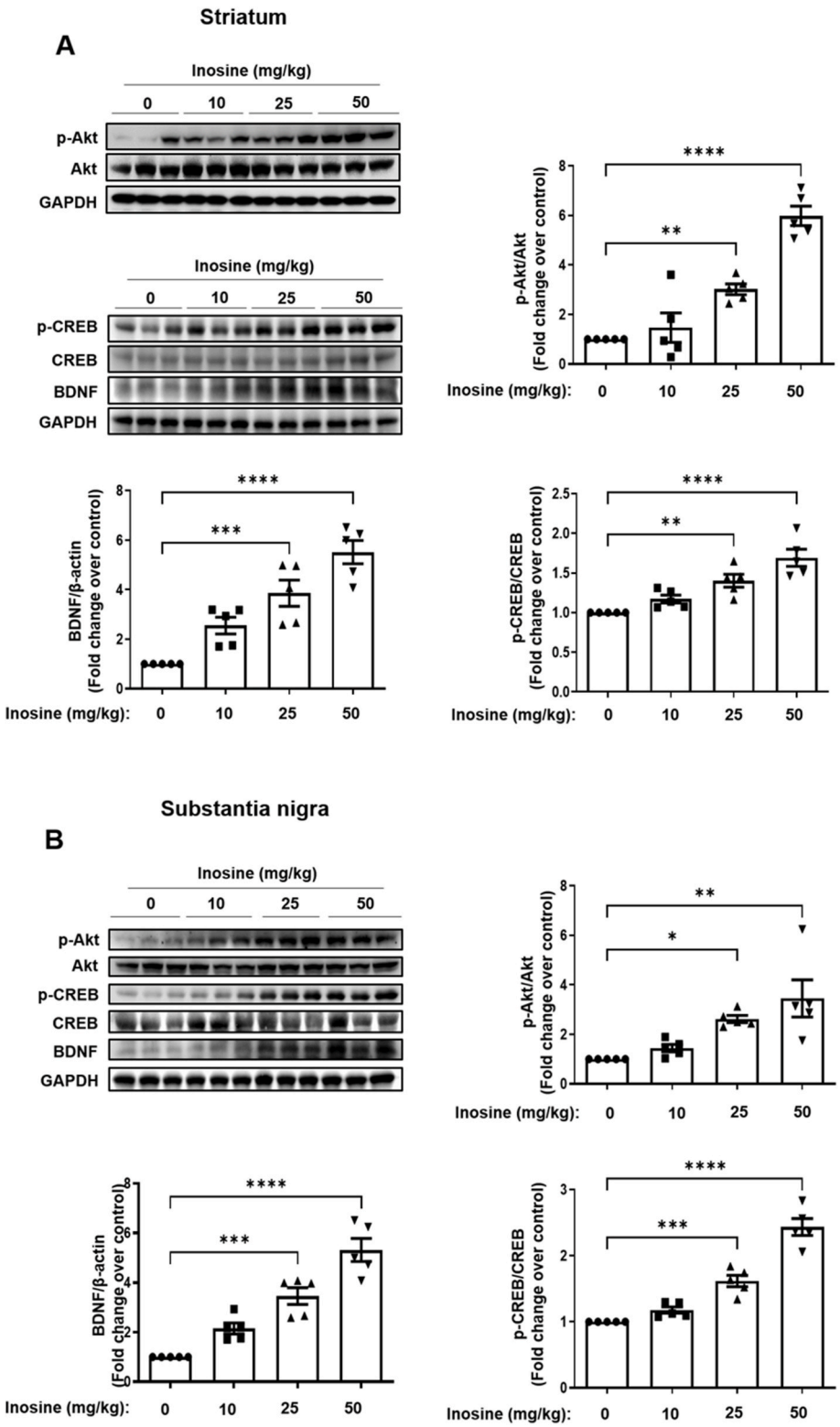

Fig. 5. Upregulation of BDNF, phospho-CREB and phospho-Akt in the striatum and substantia nigra of mice after inosine treatment.
Animals received injection of inosine (10, 25 and 50 mg/kg/day) for four weeks and then western blots were performed to assess the expression levels of BDNF, CREB and Akt in the mice brain. (A) Results of Western blot for BDNF, CREB and Akt in the striatum. (B) Results of Western blot for BDNF, CREB and Akt in the substantia nigra. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. All values represent mean ± S.E.M. (n = 5).

cells were pretreated with an A_1 receptor antagonist (DPCPX), an A_{2A} receptor antagonist (SCH58261), and an A_{2B} receptor antagonist (PSB1115) for 1 h, followed by treatment with inosine for 2 h and MPP^+ for 24 h. Cell viability tests revealed that the A_1 and A_{2A} receptor antagonists partially suppressed the cytoprotective effects of inosine ($p = 0.0142$, $p = 0.0367$) ($F_{(9, 20)} = 3$, $p < 0.0001$) (Fig. 4A). In addition, Western blot analysis revealed that BDNF reduction in the presence of MPP^+ was restored by inosine, and the restorative action of inosine was significantly attenuated by DPCPX ($p = 0.0270$) and SCH58261 ($p = 0.0037$) ($F_{(9, 20)} = 2$, $p < 0.0001$) (Fig. 4B). Overall, these results indicated a putative role of adenosine A_1 and A_{2A} receptors in BDNF upregulation and the consequent neuroprotection by inosine in SH-SY5Y cells.

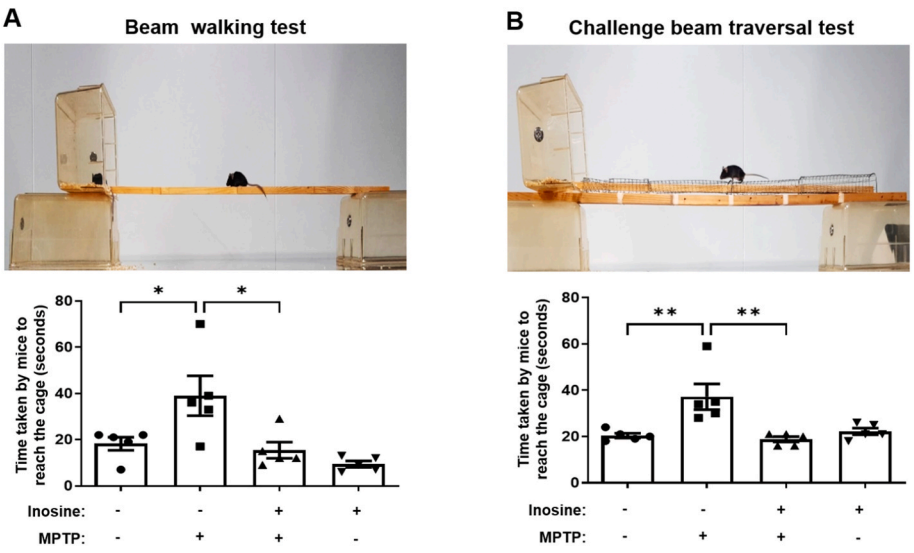

Fig. 6. Attenuation of MPTP-mediated behavioral impairment by inosine.
Animals received injection of inosine (50 mg/kg/day) for three weeks and then MPTP (15 mg/kg, 4 times). The animals were subject to the beam walking test and challenge beam traversal test to determine if inosine could attenuate deficit in behavior caused by MPTP challenge. (A) Experimental results of the beam walking test showing attenuating effect of inosine on behavioral impairment exerted by MPTP. (B) Experimental results of the challenge beam traversal test showing attenuating effect of inosine on behavioral impairment exerted by MPTP. $*p < 0.05$, $**p < 0.01$. All values represent mean ± S.E.M (n = 5).

3.4. Inosine upregulates BDNF and activates its downstream signaling pathway in the striatum and substantia nigra of C57BL/6 mice

After the *in vitro* study, we investigated whether inosine upregulates BDNF and its downstream signaling pathways in the mouse brain. C57BL/6 mice were administered different doses of inosine (10, 25, and 50 mg/kg) for 21 days, and the expression levels of BDNF and its downstream signaling molecules in the striatum and substantia nigra were analyzed. Inosine upregulated BDNF remarkably ($F_{(3,16)} = 2$, $p < 0.0001$, see Fig. 5A) and subsequently increased the activity of its downstream signaling pathway, p-Akt ($F_{(3,16)} = 3$, $p < 0.0001$, see Fig. 5A) and p-CREB ($F_{(3,16)} = 1$, $p < 0.0001$, see Fig. 5A) in the striatum and in the substantia nigra (($F_{(3,16)} = 36.48$, $p < 0.0001$, see Fig. 5B, BDNF), ($F_{(3,16)} = 8.295$, $p = 0.0015$, see Fig. 5B, p-Akt), ($F_{(3,16)} = 62.86$, $p < 0.0001$, see Fig. 5B, p-CREB).

3.5. Inosine improves the behavioral deficit caused by the MPTP treatment in C57BL/6 mice

Beam-walking and challenge beam traversal tests were performed to determine whether inosine could improve the motor deficits caused by the MPTP challenge. In the beam-walking test, MPTP injection significantly increased the time to cross the beam in comparison to the control ($p = 0.0392$). Inosine injection ameliorated the impairment induced by MPTP ($p = 0.0177$) ($F_{(3,16)} = 6.831$, $p = 0.0036$) (Fig. 6A). Similarly, the challenge beam test revealed that the mice in the MPTP group took a longer time than that taken by the control animals ($p = 0.0055$), and inosine significantly improved behavioral impairment ($p = 0.0025$) ($F_{(3,16)} = 8$, $p = 0.0017$) (Fig. 6B). The inosine treatment group exhibited no significant change in the time taken by mice when compared to the normal saline treatment group.

3.6. Inosine rescues the nigrostriatal dopaminergic neurons from MPTP-mediated injury

We performed immunohistochemical staining for TH using brain sections containing the substantia nigra and striatum to assess the extent of dopaminergic neuronal loss caused by MPTP and the rescuing properties of inosine. Immunostaining revealed that MPTP caused an extensive loss of TH-positive neurons and their terminals in the substantia nigra ($p < 0.0001$) ($F_{(3,16)} = 53.79$, $p < 0.0001$) and striatum ($F_{(3,16)} = 5$, $p < 0.0001$), respectively. The inosine regimen before MPTP injection significantly prevented neuronal loss ($p = 0.0098$) (Fig. 7A) ($p = 0.0005$) Fig. 7B). Inosine did not have any significant effect on the population of dopaminergic neurons.

Next, we conducted HPLC analysis to measure the levels of striatal dopamine and its metabolite DOPAC. The results revealed that inosine itself did not affect striatal dopamine and DOPAC levels compared to those in the normal saline group. MPTP injection caused a dramatic loss of dopamine (~65%) ($p < 0.0001$) and DOPAC (~80%) ($p < 0.0001$) in the striatum, and inosine reversed the depletion ($p = 0.0244$ and $p = 0.0027$) ($F_{(3,16)} = 2$, $p < 0.0001$, Fig. 7C; $F_{(3,16)} = 1$, $p < 0.0001$, Fig. 7D).

Next, we measured BDNF protein levels in the substantia nigra and striatum by western blotting. The results indicated that MPTP decreased neurotrophic factors in both the substantia nigra ($p = 0.0297$) and striatum ($p = 0.0003$), which was attenuated by inosine pretreatment ($p = 0.0019$ and $p = 0.0018$). In parallel with these results, the phosphorylation levels of Akt and CREB were diminished by MPTP ($p = 0.0021$, Fig. 8B; $p = 0.0004$, Fig. 8E and $p = 0.0482$, Fig. 8C; $p = 0.0032$), and inosine counteracted the action of MPTP ($p = 0.0019$, Fig. 8A; $p < 0.0001$, Fig. 8B; $p = 0.0234$, Fig. 8C; $p = 0.0018$, Fig. 8D; $p = 0.0046$, Fig. 8E; $p = 0.0020$, Fig. 8F). Importantly, inosine upregulated BDNF ($p < 0.0001$, Fig. 8A; $p = 0.0004$, Fig. 8D) and activated its downstream signaling pathway ($p < 0.0001$, Fig. 8B; $p = 0.0066$, Fig. 8C; $p < 0.0001$, Fig. 8E; $p < 0.0001$, Fig. 8F) in both the substantia nigra and striatum ($F_{(3,16)} = 38.98$, $p < 0.0001$, Fig. 8A; $F_{(3,16)} = 118.9$, $p < 0.0001$, Fig. 8B; $F_{(3,16)} = 15.34$, $p < 0.0001$, Fig. 8C; $F_{(3,16)} = 38.76$, $p < 0.0001$, Fig. 8D; $F_{(3,16)} = 70.15$, $p < 0.0001$, Fig. 8E; $F_{(3,16)} = 3$, $p < 0.0001$, Fig. 8F).

3.7. Inosine ameliorates the activation of microglia and astrocytes evoked by MPTP challenge

As neuroinflammation may contribute to the pathogenesis of PD, we investigated whether inosine could suppress neuroinflammatory responses in the striatum and substantia nigra. Immunostaining for Iba1 showed a dramatic increase in microglial activation in the substantia nigra ($p < 0.0001$) and striatum ($p < 0.0001$) of MPTP-injected mice, indicating that the cell bodies were larger and the processes were thicker and shorter than those of the control. Treatment with inosine ameliorated MPTP-induced microglial activation ($p = 0.0005$, Fig. 9A; $p = 0.0009$, Fig. 9B) ($F_{(3,16)} = 24.69$, $p < 0.0001$, Fig. 9A; $F_{(3,16)} = 38.5$, $p < 0.0001$, Fig. 9B). The results of morphological observations were further confirmed by the assessment of optical density.

Next, we analyzed the extent of astrocyte activation in the striatum and substantia nigra using immunohistochemical staining for GFAP. The results revealed that MPTP injection caused the activation of astrocytes,

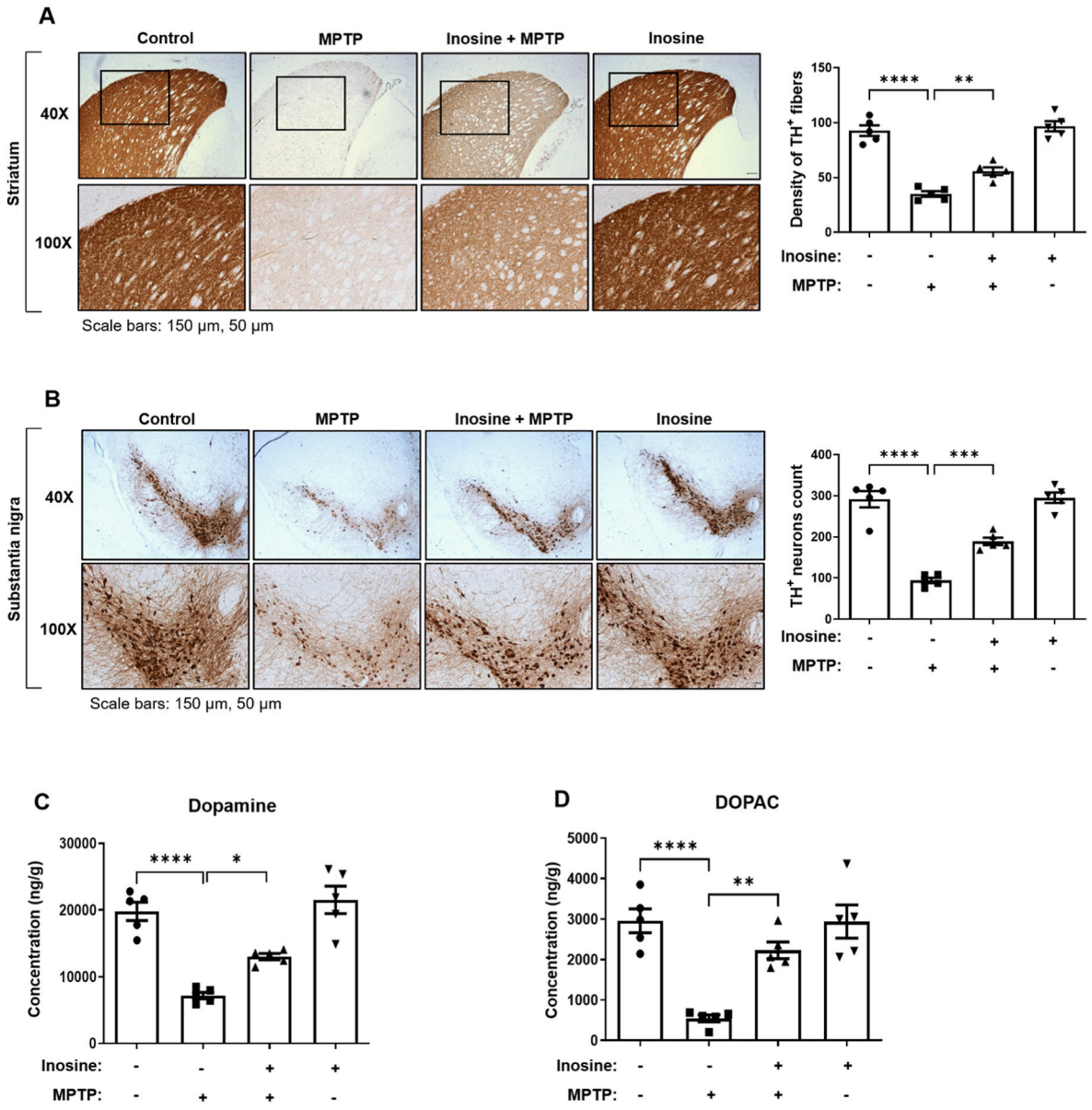

Fig. 7. Attenuation of MPTP-mediated dopaminergic neurodegeneration by inosine.
Immunohistochemical stainings for tyrosine hydroxylase were performed to assess the extent of the dopaminergic neuronal injury mediated by MPTP. Further, neurochemical analyses for dopamine and DOPAC using HPLC were conducted with tissues of striatum. (A) Results of immunohistochemical stainings for tyrosine hydroxylase in the striatum. (B) Results of immunohistochemical stainings for tyrosine hydroxylase in the substantia nigra. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. All values represent mean ± S.E.M (n = 5). Scale bars: 150 μm (40x) and 50 μm (100x).

as the number and thickness of the processes of astrocytes were clearly elevated in the substantia nigra pars compacta ($p < 0.0001$) and striatum ($p < 0.0001$). Inosine injection attenuated the MPTP-induced activation of astrocytes in the brain ($p < 0.0001$, Fig. 9C; $p = 0.0002$, Fig. 9D) ($F_{(3,16)} = 1$, $p < 0.0001$, Fig. 9C; $F_{(3,16)} = 50.99$, $p < 0.0001$, Fig. 9D). This observation was confirmed by measuring the optical density in the substantia nigra and striatum.

4. Discussion

Despite extensive research on the pathogenesis and therapeutic approaches for the treatment of PD, there is currently no known cure for the disease. A putative disease-modifying approach for PD could be to enhance the BDNF levels in the brain, as the promising neuroprotective effect of neurotrophic factors has already been demonstrated in multiple studies (Chen et al., 2013b; Almeida et al., 2005; Hetman et al., 1999). Previous studies have shown that inosine induces BDNF through adenosine receptors (Muto et al., 2014) and ameliorates neurological disorders such as major depressive disorder and Alzheimer's disease (Teixeira et al., 2020, 2022). Herein, we clearly showed that inosine rescues dopaminergic neurons from MPP^{+} and MPTP-induced injury via BDNF upregulation. A_1 and A_{2A} receptors, but not A_{2B} receptors, seemed to be involved in BDNF induction and neuroprotection, as blocking A_1 and A_{2A} receptors using their antagonists partially counteracted the pharmacological actions of inosine.

BDNF can promote mesencephalic neuron survival and neurite development (Howells et al., 2000b; Hyman et al., 1991). The neuroprotective effect of BDNF is mediated by the binding of BDNF to TrkB receptors, which initiates a cascade of downstream signal transduction pathways including PI3K, Akt, and CREB (Chen et al., 2013a; Zhong et al., 2019; Numakawa et al., 2010). CREB activity has been implicated in various neuronal physiological processes, including proliferation,

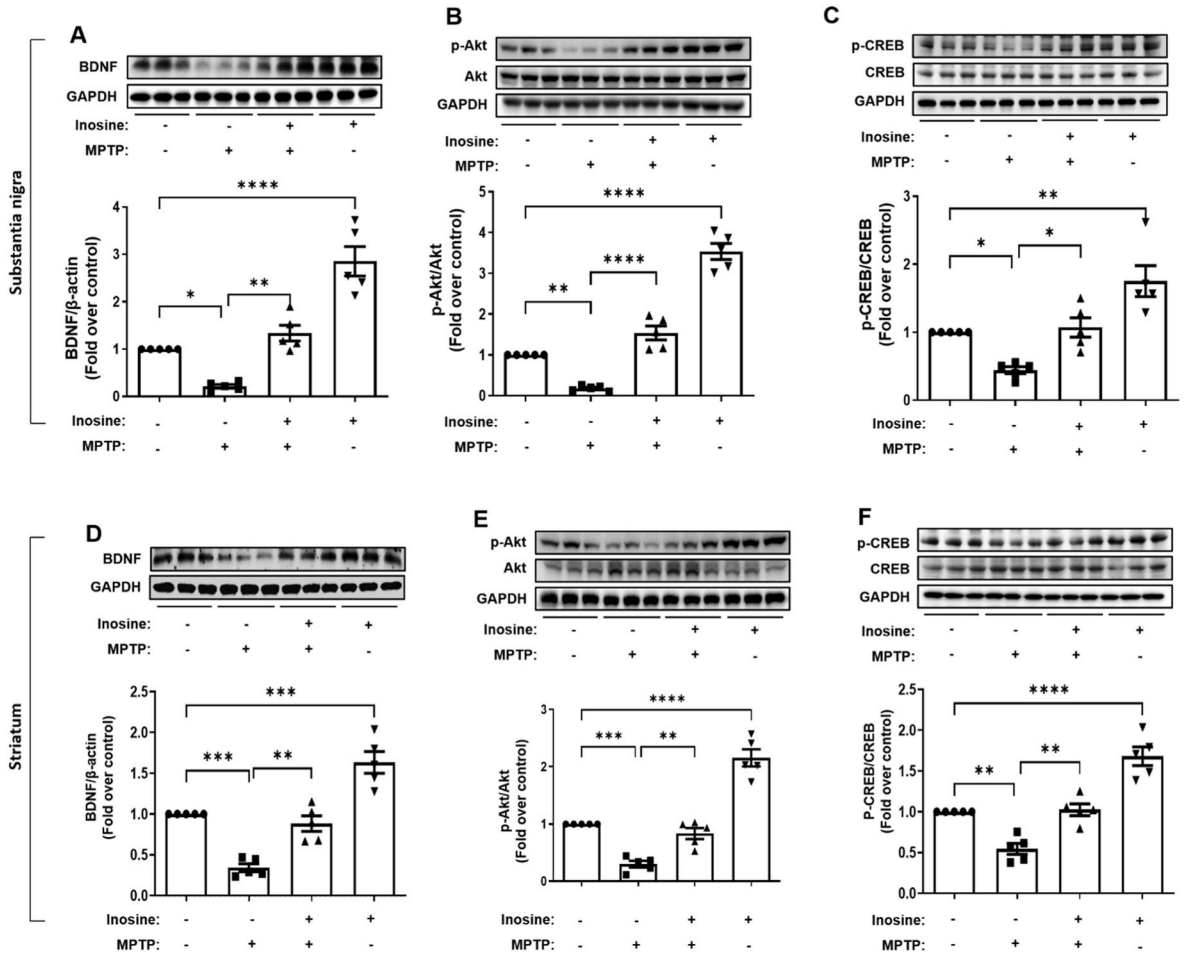

Fig. 8. Restoration of BDNF, phospho-CREB, phospho-Akt levels by inosine.
Western blots were performed to assess the levels of BDNF, Akt and CREB in the substantia nigra or striatum. (A) Effects of inosine on Akt and phospho-Akt levels in the presence or absence of MPTP challenge in the substantia nigra. (B) Effects of inosine on CREB, phospho-CREB and in the presence or absence of MPTP challenge in the substantia nigra. (C) Effects of inosine on BDNF in the presence or absence of MPTP challenge in the substantia nigra. (D) Effects of inosine on BDNF in the presence or absence of MPTP challenge in the striatum. (E) Effects of inosine on Akt and phospho-Akt in the presence or absence of MPTP challenge in the striatum. (F) Effects of inosine on CREB, phospho-CREB in the presence or absence of MPTP challenge in the striatum. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. All values represent mean ± S.E.M (n = 5).

differentiation, longevity, long-term synaptic potentiation, and neurogenesis (Wang et al., 2018). The PI3K/Akt signaling pathway is known to promote neuronal survival (Hossini et al., 2016). In line with previous studies, the present study showed that inosine upregulated BDNF and increased phosphorylation of CREB and Akt, which was related to the protective action of inosine. Importantly, the results of the *in vitro* study appeared to be recapitulated in the MPTP-induced PD model.

In a previous study, El-Shamarka et al. evaluated neuroprotective effects of inosine on 3-nitropropionic acid (3-NP)-caused neurotoxicity in rats to elucidate role of inosine in Huntington's disease (El-Shamarka et al., 2022). They found that inosine significantly attenuated 3-NP-induced loss of the striatal neurons. Importantly, they demonstrated that A_{2A} receptor-BDNF-TrkB-CREB signaling pathway is associated with the neuroprotective activity of inosine. We employed *in vitro* and *in vivo* models for PD including MPP^{+}-exposed SH-SY5Y cellular model and MPTP-treated mice model to assess the neuroprotective action of inosine. Similar to their results, we observed A_{2A} receptor-BDNF-TrkB-CREB signaling pathway plays a critical role in the inosine's neuroprotective action against MPP^{+} or MPTP-induced neurotoxicity. Aforementioned findings indicate similarity of our results to those of El-Shamarka et al. However, ours are different from theirs in several senses. First, we found that both activation of Akt and ERK, the upstream of CREB are required for the neuroprotective action of inosine. Second, we extensively investigated role of adenosine receptors in the BDNF induction and neuroprotection by employing inhibitors of A_1, A_{2A}, and A_{2B} receptor. Our results indicate that A_1 and A_{2A} receptors, but not A_{2B} play critical roles in the pharmacological action of inosine. Third, we clearly showed that BDNF is a major contributor to the neuroprotection of inosine by employing siRNA to BDNF gene, but they did not provide direct evidence of the role of BDNF in the neuroprotection.

Inosine can cross the BBB and bind to the A_1, A_{2A}, A_{2B} and A_3 receptors, acting as agonists for these receptors (Hou et al., 2004). The activation of A_1 and A_{2A} receptors may be associated with neuromodulatory effects. The A_1 receptor is found on the membranes of neurons and glia and exerts various biological actions, such as anti-seizure and sedative effects (Paul et al., 2011). The function of the A_{2A} receptor is not clear, but A_{2A} receptor is reported to be populated in the basal ganglia (Schiffmann et al., 2007). A previous study reported that antagonism of the A_{2A} receptor attenuated the motor deficit induced by neurotoxins in PD (Ikeda et al., 2002). Inosine exhibits antidepressant activity in forced swimming and tail suspension tests via activation of adenosine A_1 and A_{2A} receptors (Kaster et al., 2013). In addition, activation of the A_{2A} receptor protects PC12 cells from hypoxia-induced cell death (Kobayashi and Millhorn, 1999). Interestingly, antagonists of the A_{2A} receptor also showed protective properties

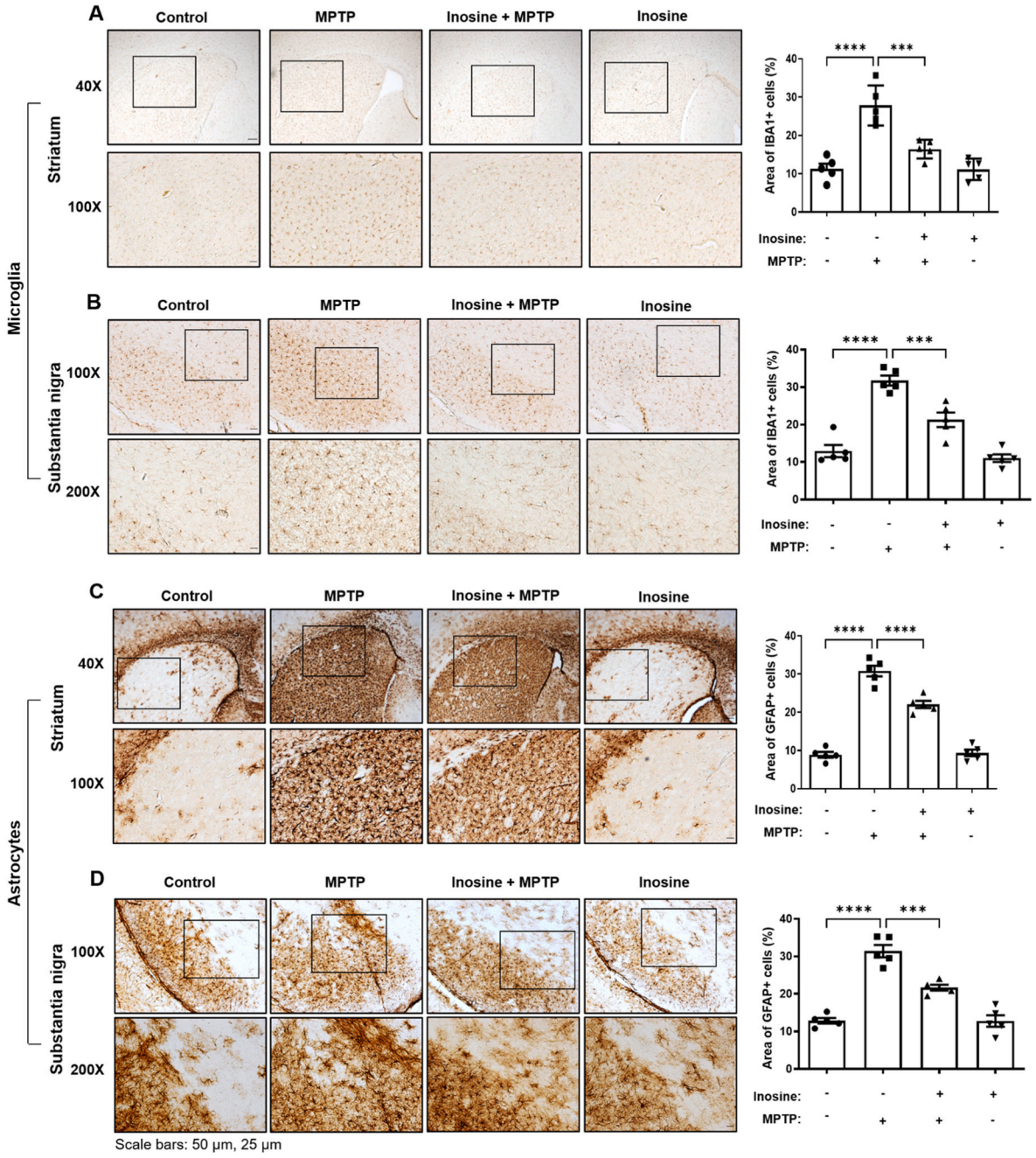

Fig. 9. Attenuation of microglial and astroglial activation by inosine.
Immunohistochemical stainings for Iba1 and GFAP were performed to assess the extent of glial activation after MPTP challenge in the striatum and substantia nigra. (A) Results of immunohistochemical stainings for Iba1 in the striatum. (B) Results of immunohistochemical stainings for Iba1 in the substantia nigra. (C) Results of immunohistochemical stainings for GFAP in the striatum. (D) Results of immunohistochemical stainings for GFAP in the substantia nigra. ***$p < 0.001$, ****$p < 0.0001$. All values represent mean ± S.E.M (n = 5). Scale bars: 150 µm (40x), 50 µm (100x) and 25 µm (200x).

in PD models (Ferreira et al., 2015). Istradefylline, an A_{2A} receptor antagonist, was approved in 2019 by the US FDA as an add-on treatment for levodopa in PD (Chen and Cunha, 2020). Istradefylline significantly reduces the off-period of PD patients by approximately 1.7 h, but with modest clinical efficacy. These findings indicate that both antagonism and agonism of the A_{2A} receptor may be beneficial to PD, depending on the symptoms or experiments.

The A_3 adenosine receptor is a Gi protein-coupled receptor abundantly expressed in the granulocytic cells and microglia, where it seems to regulate chemotaxis and immune cell activity (Fisher et al., 2022). Thus, inosine displays immunomodulatory activity in multiple experimental settings where inflammation-mediated cellular injuries occur. For instance, continuous subcutaneous infusion of inosine for six weeks reduces the infiltration of macrophages and microglia into spinal cord injury sites (Conta and Stelzner, 2008). Inosine ameliorates the release of inflammatory cytokines and protects against LPS-induced endotoxemia (Haskó et al., 2000; Lovászi et al., 2021), liver damage (Guo et al., 2021) and lung injury (Mao et al., 2022). In this context, our study showed that inosine pretreatment significantly reduced glial activation after MPTP injection (Fig. 8A–D) suggesting that the anti-inflammatory property of inosine could contribute to protection from MPTP-induced dopaminergic neuronal injury.

Although the pathogenic mechanism by which neurons are degenerated in the nigrostriatal pathway of PD brains remains obscure,

oxidative stress, mitochondrial dysfunction, and neuroinflammation are purportedly associated with the disease. Therefore, a compound with pleiotropic properties, rather than one with simple pharmacological effects, would be suitable for PD treatment. In this context, inosine is a promising candidate because it exhibits multiple pharmacological activities, including BDNF induction, anti-inflammation, anti-oxidation, and mitochondrial protection (Grünewald et al., 2019; Haskó et al., 2004; Jurkowitz et al., 1998; Bhattacharyya et al., 2016; Nascimento et al., 2021). Moreover, a clinical study showed that inosine slows the progression of PD symptoms, as determined by the UPDRS scale, which may be attributable to the antioxidant effects of uric acid (Investigators, 2014).

In conclusion, our results provide pharmacological evidence that inosine exerts neuroprotection in MPP^+/MPTP-induced neurotoxicity by activating the BDNF/TrkB signaling pathway via adenosine A_1 and A_{2A} receptor activation. Thus, inosine may be a therapeutic candidate for the treatment of PD.

Author contribution statement

Shristi Khanal: Performed experiments and analyses, collected the data, Writing- original draft, review and editing. **Eugene Bok:** investigation, and analysis. **Jaekwang Kim:** Conceived and designed the study. **Gyu Hwan Park:** Conceived and designed the study. **Dong-Young Choi:** Conceived and designed the study, Writing- original draft, review and editing, supervised the research.

Declaration of competing interest

The authors state that there are no any financial or any interpersonal conflict of interest that might have appeared to affect the research presented in this study.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2023.109652.

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