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Full Length Article

Mechanistic comparison between MPTP and rotenone neurotoxicity in mice

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ABSTRACT

Animal models for Parkinson's disease (PD) are very useful in understanding the pathogenesis of PD and screening for new therapeutic approaches. 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP) and rotenone are common neurotoxins used for the development of experimental PD models, and both inhibit complex I of mitochondria; this is thought to be an instrumental mechanism for dopaminergic neurodegeneration in PD. In this study, we treated mice with MPTP (30 mg/kg/day) or rotenone (2.5 mg/kg/day) for 1 week and compared the neurotoxic effects of these toxins. MPTP clearly produced dopaminergic lesions in both the substantia nigra and the striatum as shown by loss of dopaminergic neurons, depletion of striatal dopamine, activation of glial cells in the nigrostriatal pathway and behavioral impairment. In contrast, rotenone treatment did not show any significant neuronal injury in the nigrostriatal pathway, but it caused neurodegeneration and glial activation only in the hippocampus. MPTP showed no such deleterious effects in the hippocampus suggesting the higher susceptibility of the hippocampus to rotenone than to MPTP. Interestingly, rotenone caused upregulation of the neurotrophic factors and their downstream PI3K-Akt pathway along with adenosine monophosphate-activated protein kinase (AMPK) activation. These results suggest that MPTP-induced dopaminergic neurotoxicity is more acute and specific in comparison to rotenone toxicity, and compensatory brain-derived neurotrophic factor (BDNF) induction and AMPK activation in the rotenone-treated brain might suppress the neuronal injury.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease, affecting 0.5–1% of the elderly population (Aguirre-Vidal et al., 2015). Apart from 10% of cases reported as familial PD, the rest of the cases are sporadic or idiopathic with no known causes (Cannon et al., 2009; Le et al., 2014). PD is a progressive neurodegenerative disease characterized by a profound loss of dopamine-producing neurons of the midbrain (specifically of the basal ganglia) along with death of several non-dopaminergic neurons resulting in its reclassification as a multicentric disease (Cicchetti et al., 2009; Johnson and Bobrovskaya, 2015). Some of the well-known and important mechanisms in the pathogenesis of PD are aging, mitochondrial defects accompanied by oxidative stress, neuroinflammation, disturbed proteostasis, and calcium mishandling (Andersen and Chinta, 2016; Moon and Paek, 2015; Schapira et al., 1990).

Mitochondria, that are an integral part of all eukaryotic cells, are involved in the production of energy via the respiratory chain, regulation of cell death, calcium metabolism and production of reactive oxygen species (ROS). Deficiency of systemic mitochondrial complex I is involved in the pathogenesis of idiopathic PD as denoted by aberrant mitochondrial forms and functions (Greenamyre et al., 1999; Moon and Paek, 2015). Death of neurons occurs mostly via a reduction in mitochondrial complex I enzyme activity leading to ATP depletion, generation of ROS, and activation of caspase 3 (Greenamyre et al., 1999; Martinez and Greenamyre, 2012).

Environmental neurotoxins are utilized widely to recapitulate the symptoms and pathology of PD in experimental animals (Cannon et al., 2009; Fleming et al., 2004). MPTP and rotenone are two of such well-known neurotoxins. Both MPTP and rotenone have the ability to cross the blood brain barrier due to their high lipophilicity (Tieu, 2011). MPTP requires metabolism by the astroglial cells into its active metabolite 1-methyl-4-phenyl pyridinium (MPP^+) before entering specifically into dopaminergic neurons through dopamine transporters, whereas rotenone needs no metabolism or transporters to enter neurons (Giordano et al., 2012; Nopparat et al., 2014). These toxins inhibit the mitochondrial complex I activity thereby increasing oxidative stress and are widely utilized in rodents and other species for mimicking experimental PD (Angelova and Abramov, 2018).

The MPTP model is the most commonly used animal model of PD, but the major limitation is the inability to produce progressive neurodegeneration and Lewy body pathology (Le et al., 2014; Schober,

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2004). Rotenone on the other hand has been shown to induce phosphorylation and aggregation of α-synuclein, Lewy body-like pathology, DJ-1 acidification and translocation, proteasomal dysfunction and nigral iron accumulation which are cardinal pathologic changes in the brains of patients with PD (Cannon et al., 2009; Duty and Jenner, 2011; Johnson and Bobrovskaya, 2015). Despite its great potential, the main problem with this toxin has been its systemic toxicity leading to high morbidity and mortality. Also, lower doses either fail or cause highly variable loss of dopaminergic neurons (Vingill et al., 2017). Some of the other limitations of this model are the location and distribution of the lesions within the striatum, the magnitude of the lesions and neurochemical deficits (Wrangel et al., 2015). These issues have been overcome to some extent by using different routes of administration (Johnson and Bobrovskaya, 2015).

In this investigation, we analyzed the difference between the subchronic effects of MPTP and rotenone on the behavioral, neurochemical, immunohistological, and biochemical parameters. This study displays that MPTP neurotoxicity is more specific and acute than rotenone toxicity, and induction of neurotrophic factor and AMP-activated protein kinase (AMPK) activation may be related to the minimal effects of rotenone on the biochemical physiology of the brain. Intriguingly, rotenone induced degeneration of the hippocampal neurons to some extent.

2. Materials and methods

2.1. Animals and treatment of toxins

Adult (10-week-old) male C57BL/6 mice (20–25 g, Japan SLC, Inc.) were housed in microisolator cages on a 12 h light/dark cycle with free access to food and water. After 1 week of acclimatization, the mice were divided into different groups.

Animal experiments were performed according to the protocol approved by the IACUC of Yeungnam University before the study (approval number: 2016-004). All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the National Institute of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Three groups containing four mice in each group were treated intraperitoneally with the vehicle, rotenone (2.5 mg/kg/day; Tokyo Chemical Industry Co.) and MPTP (30 mg/kg/day; Sigma-Aldrich) for 6 days. Each mouse was euthanized by CO_2 asphyxiation on Day 6. For each animal, the brain tissues were dissected, snap frozen on dry ice, and stored at -80 °C until analysis.

2.2. Determination of neurochemicals using high-performance liquid chromatography

High-performance liquid chromatography (HPLC) (1260 Infinity, Agilent technologies) for the measurement of dopamine (DA) and its metabolite 3, 4-dihydroxyphenylacetic acid (DOPAC) was performed. Briefly, striatal tissue samples were weighed, transferred into 100 µl of 0.1 N perchloric acid (PCA), and sonicated until homogenization. After centrifugation at 13,000 *g* at 4 °C for 20 min, the supernatants were collected for chromatographic assay. Also, standard solution for DA and DOPAC was made in 0.1 M PCA and a series of standard solutions were prepared freshly by diluting the stock solution in PCA. 10 µl of prepared supernatant or standard solution was injected into the mobile phase (75 mM sodium phosphate monobasic, 1.7 mM 1-octanesulfonic acid, 100 µl/L Triethylamine, 25 µM EDTA, 15% acetonitrile) with the flow rate of 0.6 ml/min. DA and DOPAC were detected by an electrochemical detector (Coulochem III, Thermo Fisher Scientific Inc.). Finally, the values were derived and the concentrations of monoamines were calculated in ng/wet tissue weight.

2.3. Western blot analysis

Brain tissue samples were homogenized with ice-cold NP-40 lysis buffer [0.15 M NaCl, 1% NP-40, 0.05 M Tris (pH 8.0), and 1% protease inhibitor cocktail (Thermo Fisher Scientific Inc.)]. The tissue homogenates were centrifuged at 4 °C for 20 min at 13,000 *g*, and the supernatant was transferred to a fresh tube. Protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific Inc.). Equal amount of protein (30 ~ 40 µg) was mixed with loading buffer (0.125 M Tris − HCl, pH 6.8, 20% glycerol, 4% sodium dodecyl sulfate (SDS), 10% mercaptoethanol, and 0.002% bromophenol blue) and boiled for 7 min to induce denaturation of proteins. The samples were separated on 12% SDS-polyacrylamide gel by electrophoresis and transblotted to polyvinylidene difluoride membranes (Millipore Corporation, Temecula, CA). Membranes were blocked using 5% skimmed milk in Tris-buffered saline (0.1% Tween 20, TBST), for 1 h. To detect phospho-proteins, the membrane was blocked in 5% bovine serum albumin in TBST. Then, the membrane was incubated overnight at 4 °C with specific primary antibodies against BDNF (1:2000; Santa Cruz), GDNF (1:2000, Abcam), AMPK (1:2000; Cell Signaling Technology), p-AMPK (1:2000; Cell Signaling Technology), phosphatidylinositol-3 kinase (PI3K; 1:2000, Cell Signaling Technology), p-PI3K (1:2000, Cell Signaling Technology), protein kinase B (Akt; 1:2000; Cell Signaling Technology), p-Akt (1:2000; Cell Signaling Technology), β-actin (1:10,000; Ab-Frontier), and GAPDH (1:10,000; Ab-Frontier). After being washed thrice with TBST, the membrane was incubated with a secondary antibody for 1 h at room temperature and again washed thrice with TBST. Finally, the membrane was incubated with enhanced chemiluminescence reagents (Thermo Scientific Inc.) and exposed in a luminescence image analyzer (Fusion Solo, VilberLourmat, France) to detect the immunoreactive complex. The density of each blot was assessed using GelQuant.Net software.

2.4. Behavioral tests

Behavioral tests were carried out on Day 0 and 5 to evaluate the motor deficits in the MPTP and rotenone-intoxicated animals. The following tests were performed.

2.4.1. Beam walking test

Beam walking test was performed as described in previous studies with minimal modification (Baranyi et al., 2016; Schintu et al., 2009). Briefly, a wooden beam (100 cm long) with four equal sections of decreasing width (starting from 3.5 cm and ending with 0.5 cm) was elevated above the floor and placed in such a way that the narrow end led to the home cage (Fig. 2). Each mouse was placed on the start point of the beam and allowed to walk along the beam towards the home cage. The animals were trained 4 or 5 times per day for 5 days, before the actual assessment. During the first or second trials, the mice were assisted by gently orienting them or touching their backside to encourage them to move along the beam and reach their home cage. Typically, within a few trials, mice were able to traverse the beam on their own without need for correcting. The beam was thoroughly cleaned between the tests with each animal to remove any urine or fecal pellets that might distract the next mouse. A video camera was used to record all beam traversal trials for each mouse. The time required to traverse from the wide end to the narrow end to reach the home cage was measured using a stopwatch to quantify the motor deficits in each animal. Three repetitive trials were performed at 5 min intervals as a recovery period after each trial. The data were presented as the mean of 3 trials/per animal.

2.4.2. Challenging beam traversal test

Mesh grids (1 cm squares) with width corresponding to the size of the beam were placed over the beam surface leaving a 1 cm space between the grid and the wooden beam surface (Fig. 2). The animals were

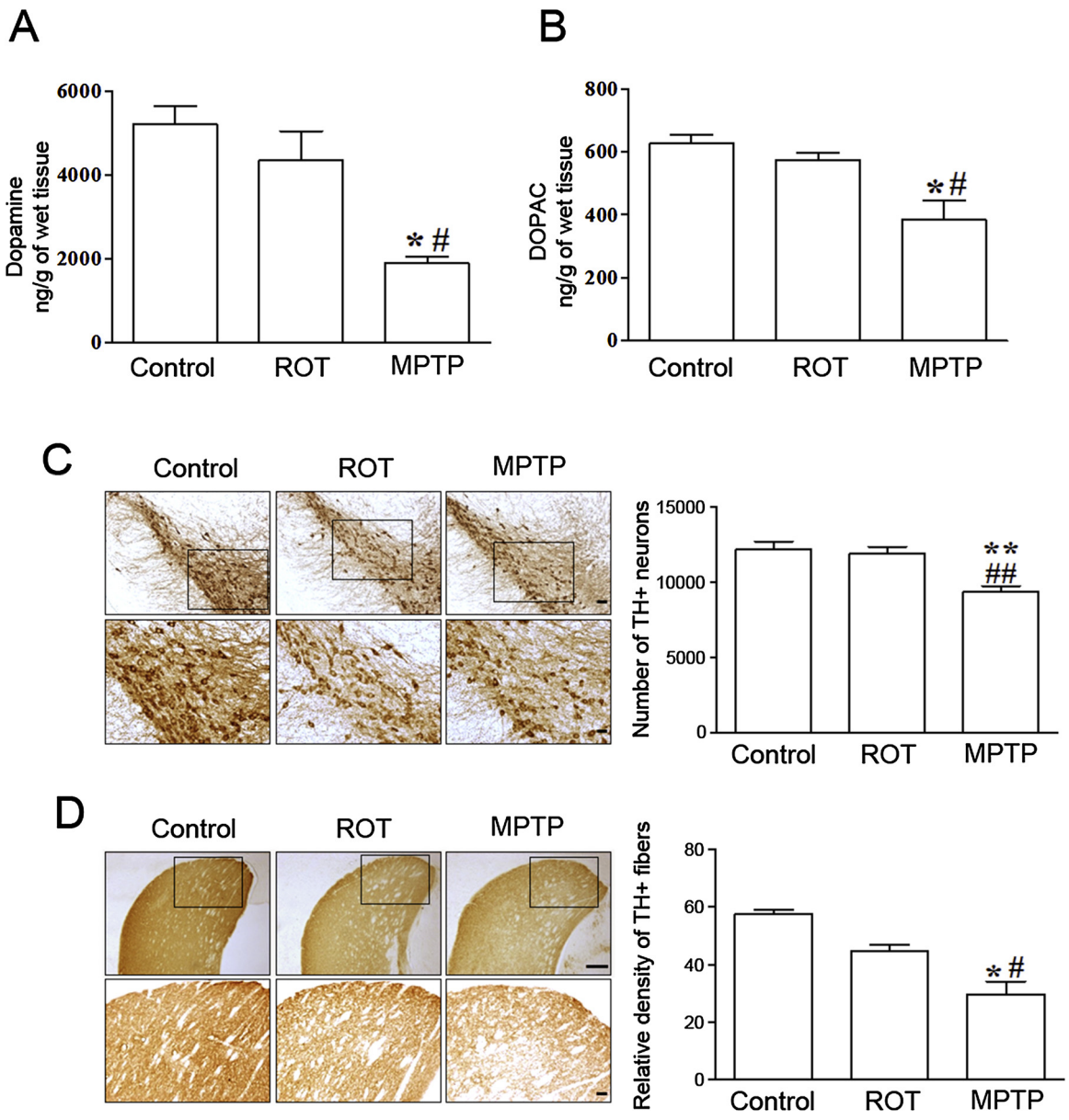

Fig. 1. Degeneration of the nigrostriatal pathway after MPTP injections.
Animals received rotenone or MPTP for 6 days and were sacrificed. Striatal concentrations of (A) DA and (B) 3,4-Dihydroxyphenylacetic acid (DOPAC) were analyzed using HPLC. Immunostaining for TH was performed with the (C) substantia nigra and (D) striatum. *$p < 0.05$, **$p < 0.01$ compared to control, #$p < 0.05$, ##$p < 0.01$ compared to the rotenone treated group (n = 4). 4× scale bar: 150 µm, 10× scale bar: 50 µm and 20× scale bar: 25 µm.

then allowed to walk over it and the time required to reach the home cage was noted, and the whole process was videotaped while animals traversed the grid-surfaced beam. The trials were repeated thrice for each mouse. The videos were later viewed in slow-motion to count the total number of foot slips per animal.

2.4.3. Cylinder test

Normally, mice explore around when kept in a new premise. Thus, when kept in a transparent cylinder, they explore within the cylinder by moving around and raising their bodies to touch the cylinder walls with their forelimbs; this activity is called rearing. This behavior is natural, but mice intoxicated with neurotoxic agents such as rotenone, MPTP, and 6-hydroxydopamine show reduced movement and rearing. The test was carried out as described in previous studies (Cannon et al., 2009; Fleming et al., 2004). Briefly, one mouse at a time was placed in a transparent glass cylinder (height = 20 cm, diameter = 12 cm) for 3 min and the process was videotaped for analysis. We counted only when the mouse raised its forelimbs above shoulder level and removed both forelimbs from the cylinder before another rearing.

2.5. Immunohistochemical staining

All mice were euthanized by CO_2 asphyxiation on the last day of the experiment. The right cerebral hemisphere was fixed in 4% paraformaldehyde for 24–36 h, and then transferred into 30% sucrose (in 1× phosphate buffered saline) and stored at 4 °C until the tissue sank in the sucrose. Coronal brain sections (30 µm thick) were cut on a freezing sliding microtome (Microm HM 450, Thermo Scientific) and the tissue sections were collected in cryoprotectant solution in 24-well plates. Immunostaining for tyrosine hydroxylase (TH)-positive cells, astroglial cells and microglial cells were performed using free-floating brain sections. Endogenous peroxidase activity of the brain sections was quenched by incubation in 3% hydrogen peroxide (H_2O_2) for 20 min. The sections were then rinsed in 0.05 mM potassium phosphate

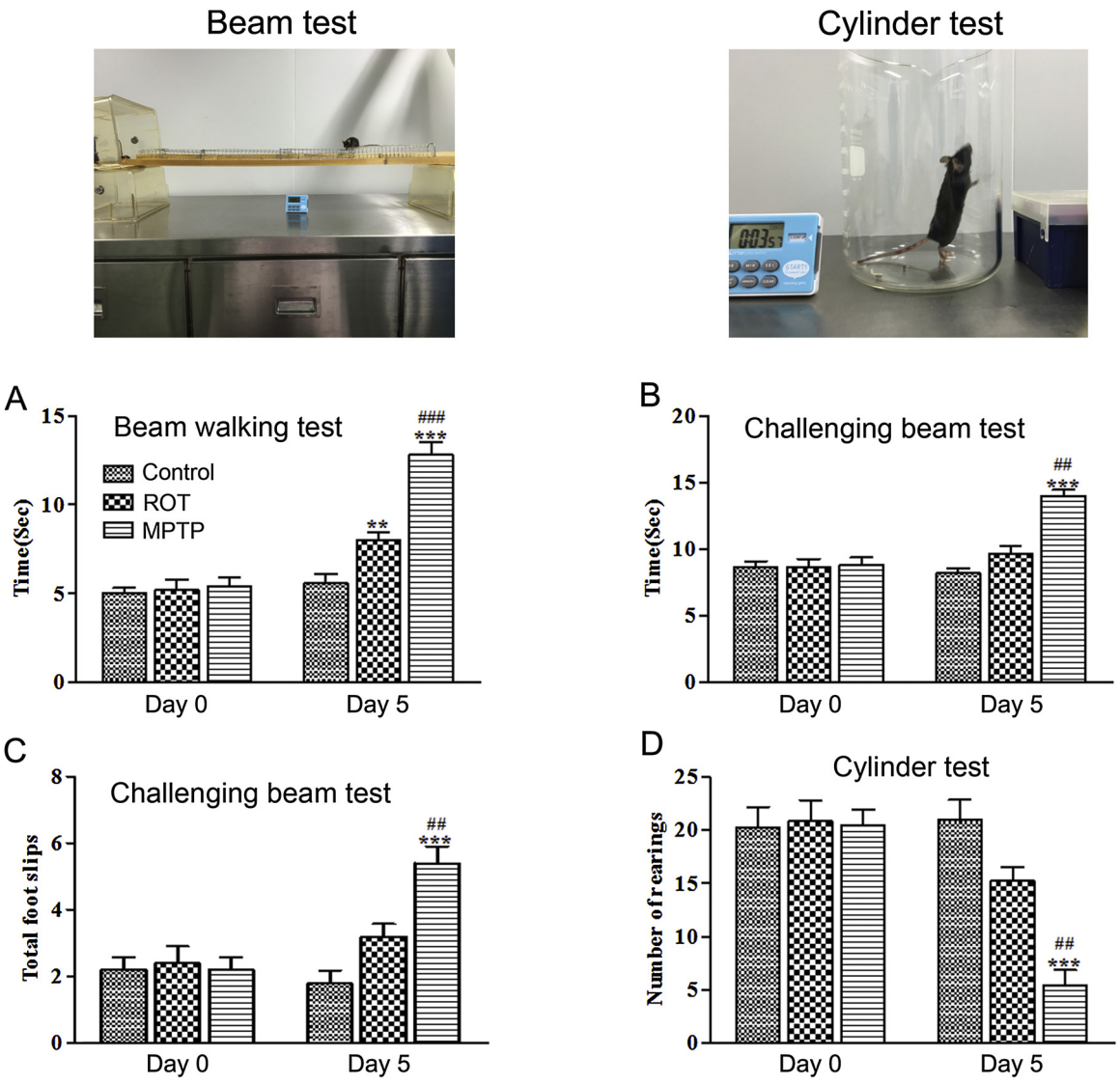

Fig. 2. Motor dysfunction caused by MPTP or rotenone.
Behavioral tests, including beam test and cylinder test, were performed 1 day before and 5 days after the initial treatment of rotenone or MPTP to evaluate the change in behavior of the animals. Data display the results of (A) beam walking test, (B) challenging beam test (walking time), (C) challenging beam test (foot slips) and (D) cylinder test, respectively. ***p < 0.001 compared to control, ##p < 0.01 ###p < 0.001 compared to the MPTP treated group. (n = 4).

buffered saline (KPBS). Sections were then incubated at 4 °C overnight with a rabbit polyclonal primary antibody against TH (1:5000, Thermo Scientific), GFAP (1:10000, Thermo Scientific), or IBA-1 (1:3000, Wako) prepared in KPBS. After rinses in KPBS, the sections were treated for 2 h in a biotinylated secondary antibody (1:1000, Vector Laboratories Inc., Burlingame, USA) diluted in KPBS containing 0.4% Triton X-100. Following washes in KPBS, the sections were immersed in avidin-biotin-peroxidase complex (Vector Laboratories Inc.) for 1 h at room temperature and rinsed in KPBS. The immunocomplex was visualized using a diaminobenzidine (Sigma-Aldrich) solution (4 mg/10 ml KPBS + 5 µl 30% H_2O_2). Sections were mounted onto gelatin-coated glass slides, left to dry overnight, dehydrated in ethanol, cleared in xylene, and cover slipped before observing under a light microscope. The number of TH-positive cells with typical neuronal morphology in the substantia nigra and the density of TH-positive fibers in the striatum were quantified by stereological count as described before (Katila et al., 2017).

The extent of glial activation in the substantia nigra and hippocampus was measured as described before. Briefly, digital images of the substantia nigra and striatum were obtained, and GFAP or IBA-1 immunoreactivity was estimated using ImageJ (http://rsbweb.nih.gov/ij/). The threshold value was adjusted to 200, and the number of pixels with a threshold more than 200 was measured. All values were displayed as a percentage of total pixels within the microscope field.

2.6. Fluoro Jade C (FJC) staining

For visualization of degenerating neurons in the hippocampus, brain sections containing the hippocampus were mounted on gelatin-coated slides, air-dried, and subjected to FJC staining. First, the slides were immersed in 100%, 95% and 70% ethanol for 5 min each. Then, they were incubated in 0.06% potassium permanganate solution for 10 min. Following rinsing with KPBS for 2 min, the slides were transferred to a 0.0001% solution of FJC dissolved in a 0.1% acetic acid vehicle. The slides were then rinsed in KPBS thrice. Excess solution was drained onto a paper towel, and the slides were then dried on a slide warmer at 50 °C. The dried slides were then cleared in xylene and then a cover slip was placed on top of each slide. Then, the sections were examined under a fluorescence microscope (Nikon, Melville, NY).

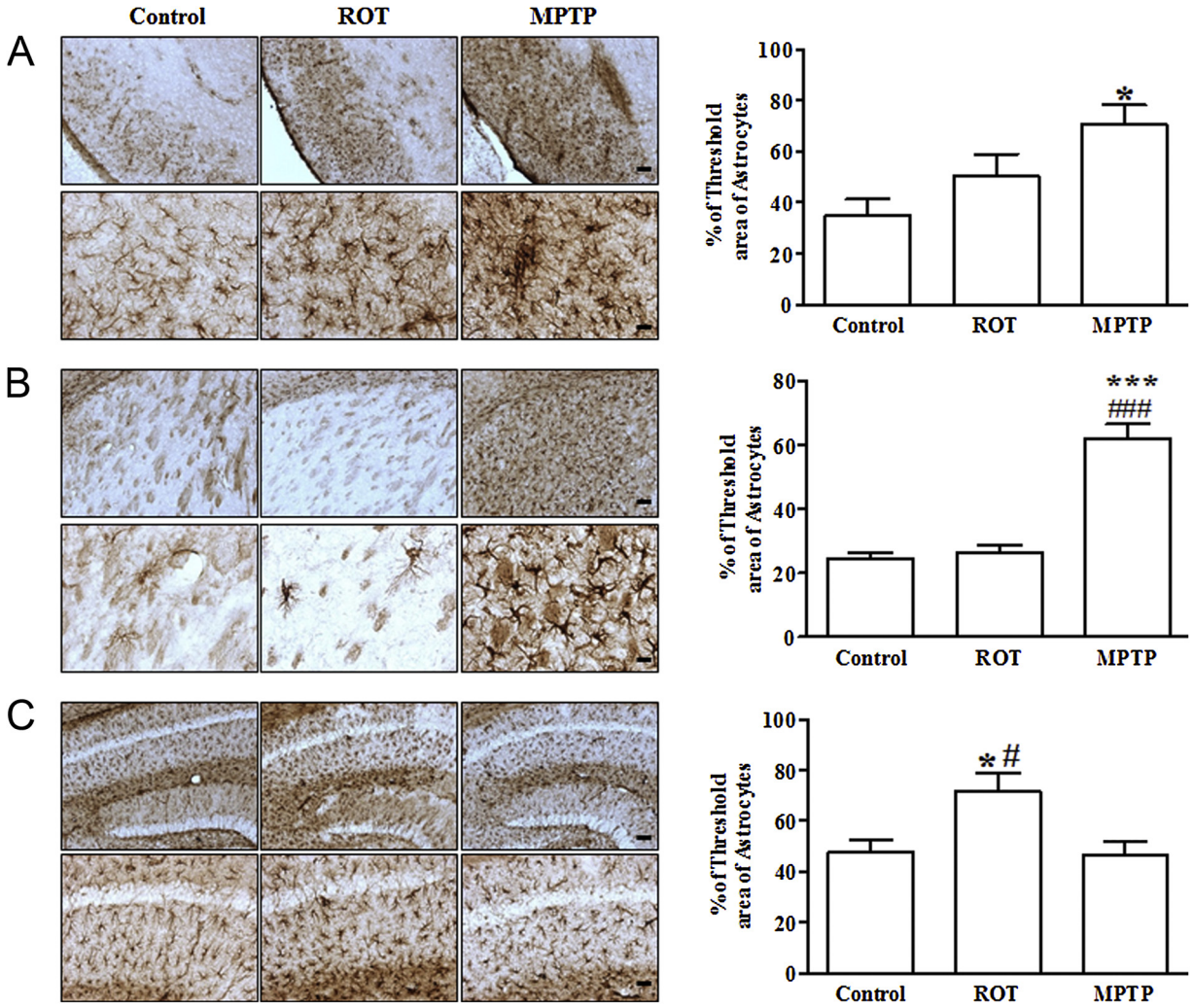

Fig. 3. Astroglial activation in the substantia nigra, striatum or hippocampus.
Immunostaining for GFAP was performed with the substantia nigra, striatal, and hippocampus sections to estimate the extent of astroglial activation. Results display the immunoreactivity for GFAP in the (A) substantia nigra, (B) striatum and (C) hippocampus. $*p < 0.05$, $***p < 0.001$ compared to control, $\#p < 0.05$, $\#\#\#p < 0.001$ compared to the rotenone treated group (n = 4). 10× Scale bar: 50 µm, 20× scale bar: 25 µm and 40× scale bar: 12.5 µm.

2.7. Statistical analysis

The data were expressed as the mean ± SEM of independent experiments. Statistical analysis was performed using one-way ANOVA followed by the Student–Newman–Keuls comparison method to calculate statistical differences between various groups (GraphPad Prism 5.0, San Diego, CA, USA). *p* values less than 0.05 were considered statistically significant.

3. Results

3.1. MPTP causes dopaminergic neurodegeneration, but not rotenone

To investigate levels of DA and its metabolite DOPAC, we performed HPLC with striatal tissue obtained from MPTP or rotenone intoxicated mice. We found that MPTP significantly reduced the DA and DOPAC levels in the striatum compared to the control and rotenone groups (Fig. 1A, B). However, there was no significant change in levels of DA and DOPAC in rotenone-treated mice (Fig. 1A, B) indicating the greater capacity of MPTP to reduce the levels of DA and its metabolite in the striatum in the subchronic model.

Next, we performed the immunohistochemical staining for tyrosine hydroxylase to visualize the dopaminergic neurons in the substantia nigra and terminals in the striatum. The results showed that MPTP caused extensive loss of dopaminergic neurons and fibers in the regions in comparison to the saline and rotenone treated group (Fig. 1C, D).

3.2. MPTP produces motor dysfunction, but not rotenone

We conducted behavioral tests including the beam tests, challenging beam tests and cylinder tests to analyze the motor deficits. The tests were performed on Days 0 and 5. The MPTP treated group took a longer time to reach the home cage in both the beam walking test (Fig. 2A) and the challenging beam test (Fig. 2B) compared to the control and rotenone groups. The number of foot slips was significantly higher in the MPTP injected group in comparison to the rest of the groups (Fig. 2C). In the cylinder test, the rearing behavior was reduced in the MPTP intoxicated mice as compared to the saline- and rotenone-treated groups on Day 5 (Fig. 2D). All these results correlated well with the MPTP-induced loss of the dopaminergic neurons as shown by immunohistochemical and HPLC analyses.

3.3. MPTP induces glial activation in the substantia nigra and striatum, but not rotenone

We performed immunostaining for GFAP and IBA-1 to determine if

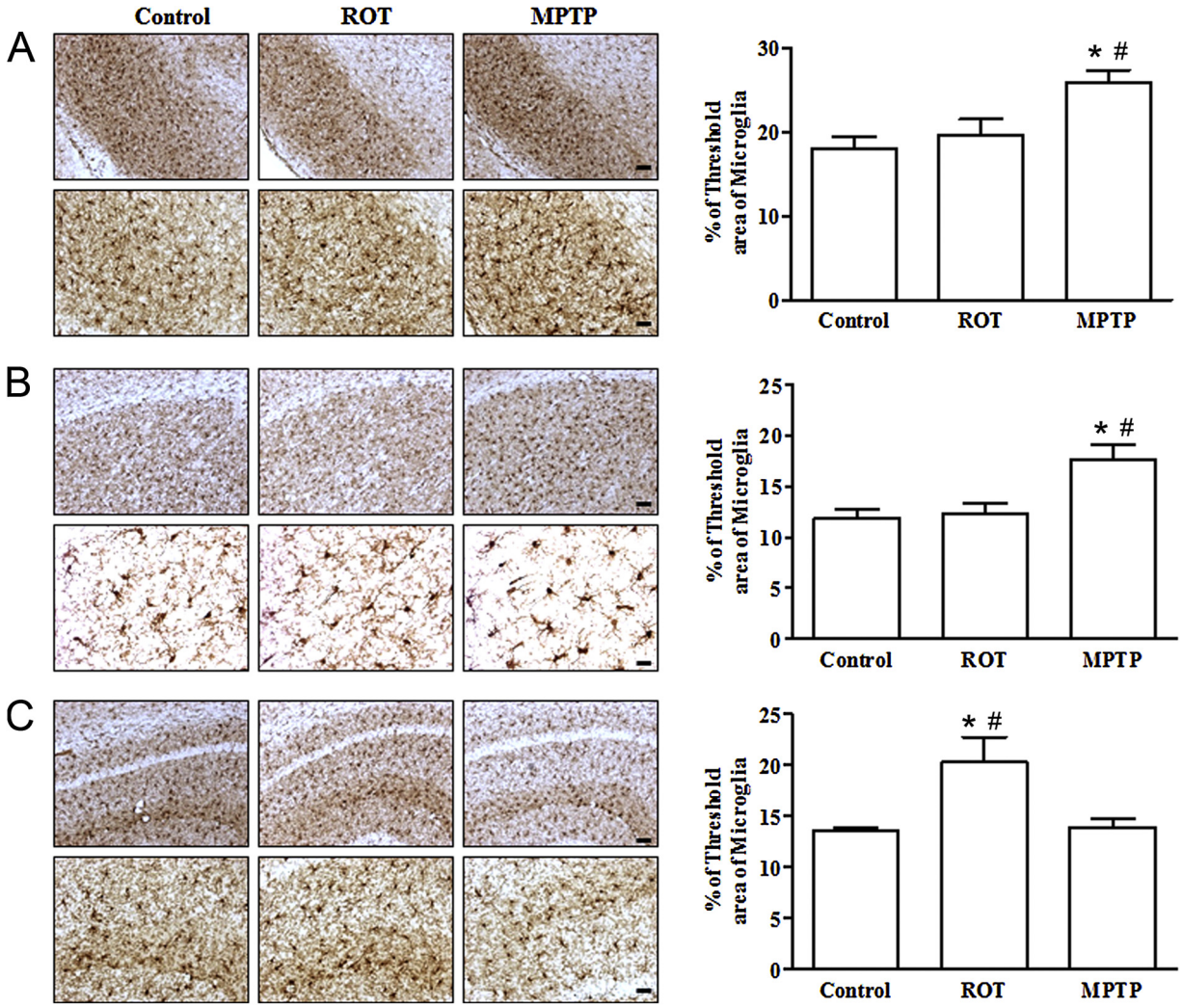

Fig. 4. Microglial activation in substantia nigra, striatum or hippocampus.
Immunostaining for IBA-1 was performed with brain sections of the substantia nigra, striatum, and hippocampus to evaluate extent of microglial activation. Results display immunoreactivity for IBA-1 in the (A) substantia nigra, (B) striatum and (C) hippocampus. *$p < 0.05$ compared to control, #$p < 0.01$ compared to the rotenone treated group (n = 4). 10× Scale bar: 50 μm, 20× scale bar: 25 μm and 40× scale bar: 12.5 μm.

the neurotoxins activated glial cells. Three regions of the brain, including the substantia nigra, striatum and hippocampus were used for immunostaining. The results showed that MPTP induced substantial astroglial activation in the substantia nigra and the striatum (Fig. 3A and B), but not in the hippocampus (Fig. 3C). Rotenone activated astrocytes in the hippocampus, but it had no significant effects on the astrocytes in the substantia nigra and striatum (Fig. 3).

IBA-1 immunostaining demonstrated microglial activation was more severe in both the substantia nigra and the striatum of MPTP treated brain than in the controls or in the rotenone-treated brain (Fig. 4A and B). In contrast, rotenone distinctly and significantly activated the hippocampal microglia (Fig. 4C), but not nigral and striatal microglia. These results indicate that the substantia nigra and striatum were selectively affected by MPTP, while the hippocampus was affected by rotenone. Further, these results suggest that there was neurodegeneration in the hippocampus after rotenone injection.

3.4. Rotenone causes degeneration of the hippocampal neurons, but not MPTP

As we observed greater activation of the glial cells in the hippocampus of rotenone-treated mice when compared to MPTP-treated mice, we speculated that rotenone might cause dengeneration of the hippocampal neurons. Hence, for the observation of degenerating neurons in the hippocampus, we utilized FJC staining, which stains all the degenerating neurons selectively, regardless of any specific insult or mechanism of cell death (Ehara and Ueda, 2009). Indeed, we observed some of FJC-positive cells in the hippocampus of control or MPTP-treated mice. Importantly, the degenerating neurons in the cornu ammonis 3 (CA3) and the dentate gyrus (DG) region of the hippocampus were more numerous in rotenone group as compared to the control and MPTP groups (Fig. 5). These findings suggest a susceptibility of the hippocampal neurons to rotenone rather than MPTP.

3.5. Rotenone potently induces neurotrophic factors and PI3K-Akt pathway

Our next aim was to evaluate the possible reason behind the inability of rotenone to cause neurodegeneration in the substantia nigra and the striatum. We carried out western blot analysis to evaluate the effects of rotenone and MPTP on the neurotrophic factors and its downstream signaling cascades such as PI3K-Akt pathway. Interestingly, rotenone significantly increased the levels of both BDNF and GDNF in the substantia nigra, but MPTP did not show any extensive changes (Fig. 6A and B).

Subsequently, we performed further investigation to determine whether the activation of downstream signaling cascades of

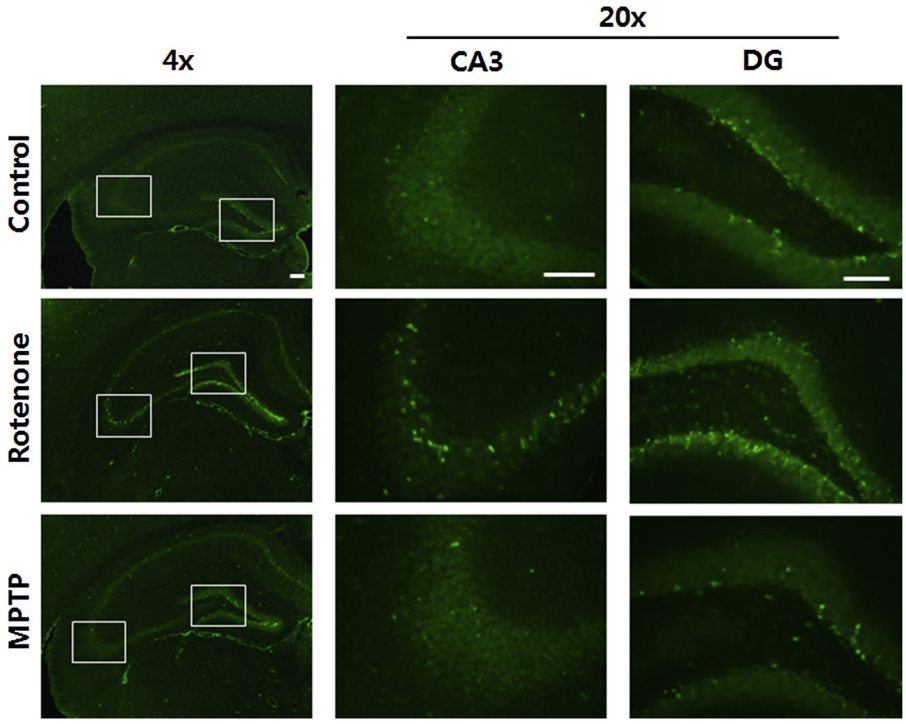

Fig. 5. Neurodegeneration in the hippocampus after rotenone treatment.
Fluoro Jade C staining was performed in hippocampus to detect the degenerating neurons. Representative photomicrographs showed that the number of FJC-positive neurons were higher in the CA3 and DG regions of hippocampus of rotenone treated mice in comparison to those of control or MPTP-treated mice (n = 4). 4× scale bar: 50 μm, 20× scale bar: 100 μm.

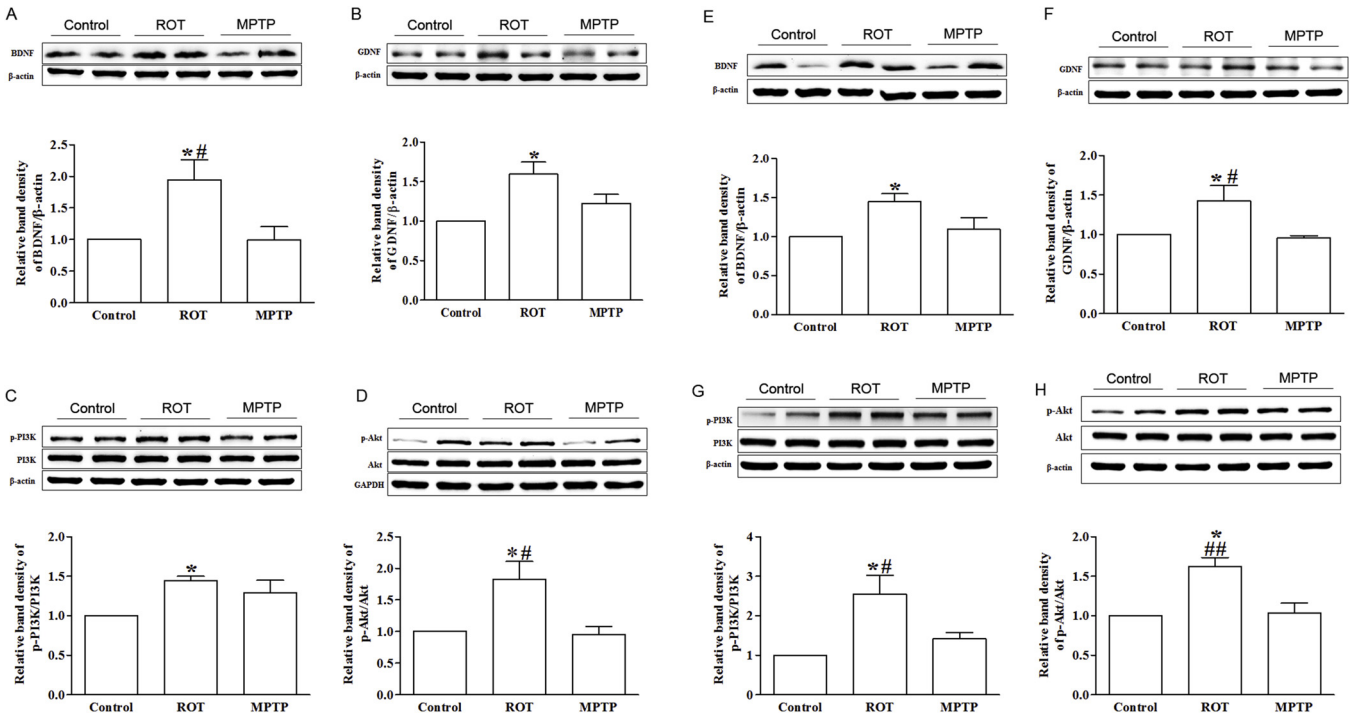

Fig. 6. Upregulation of neurotrophic factors and its downstream signaling pathways by rotenone.
Western blotting was performed to evaluate the levels of BDNF, GDNF, PI3K and Akt in the substantia nigra and the hippocampus. Results show immunoblots for (A) BDNF, (B) GDNF, (C) PI3K and (D) Akt in the substantia nigra, and (E) BDNF, (F) GDNF, (G) PI3K and (H) Akt in the hippocampus, respectively. $*p < 0.05$ compared to control, $\#p < 0.05$, $\#\#p < 0.01$ compared to the MPTP treated group. (n = 4).

neurotrophic factors followed the above trend. Indeed, we observed that the pattern correlated with the neurotrophic factor levels in the specific regions such that treatment with rotenone caused a significant increment in the phosphorylation of PI3K and Akt in the substantia nigra, while treatment with MPTP caused a slight increment of PI3K only but had no any effect on the Akt (Fig. 6C and D). Similar results were obtained in the hippocampus. Rotenone resulted in the significant induction of BDNF and GDNF (Fig. 6E and F) followed by the increased phosphorylation of the PI3K-Akt pathway compared to the saline- and MPTP-treated groups (Fig. 6G and H).

3.6. Rotenone increases AMPK phosphorylation

We also tested the phosphorylation level of AMPK in the substantia

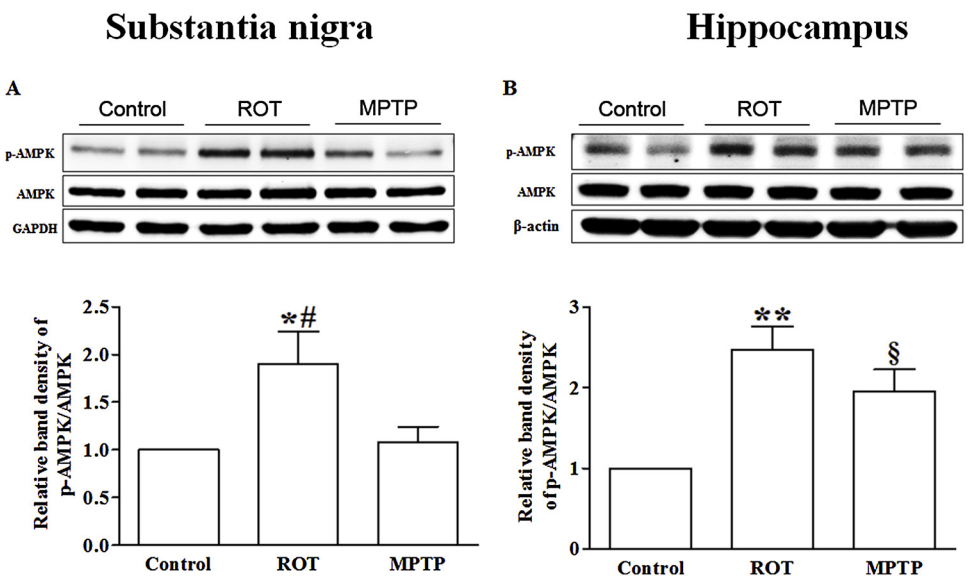

Fig. 7. Increase in AMPK phosphorylation after rotenone treatment.
Western blotting was performed to check the levels of AMPK and phospho-AMPK in the substantia nigra and hippocampus. Results show immunoblots for (A) AMPK and phospho-AMPK in the substantia nigra, and (B) AMPK and phospho-AMPK in the hippocampus, respectively. $*p < 0.05$, $**p < 0.01$ compared to control, $\#p < 0.05$ compared to the MPTP treated group. (n = 4).

nigra and hippocampus (Fig. 7A and B) to elucidate the effect of these toxins on AMPK activity since AMPK is an important energy sensor in living cells (Gomez-Pinilla et al., 2008). Interestingly, rotenone significantly increased the AMPK phosphorylation in the hippocampus and substantia nigra, but MPTP had no effect in these brain regions.

These results suggest that in the substantia nigra, upregulation of neurotrophic factors, their signaling molecules, and AMPK activation might play a major role in combating the neurotoxic effects of rotenone, thereby resulting in its inability to cause any neurodegeneration. But in the hippocampus, increased glial activation, NTFs, and AMPK phosphorylation might be associated with a protective mechanism guarding this region against the neurotoxin.

4. Discussion

In this study, we aimed to compare the effects of the two commonly used neurotoxins, MPTP and rotenone. We primarily focused on the nigrostriatal pathway that undergoes extensive degeneration in PD together with the hippocampus, which is an important part involved in learning and memory and which is also affected in PD (Anand and Dhikav, 2012; Hall et al., 2014). We observed that for the subchronic model, MPTP is a better and more reliable neurotoxin when compared to rotenone in producing the nigrostriatal lesion, the depletion of dopamine and the activation of glial cells. One important finding of our research was that rotenone significantly upregulated neurotrophic factors in the brain which might be the possible reason for the failure of rotenone to produce features of PD in the short-term model. Interestingly, rotenone distinctly caused glial activation and loss of the hippocampal neurons.

Experimental animal models have undoubtedly been a useful tool not only when it comes to understanding the pathogenesis of human diseases but also to enable us to search for novel therapeutic interventions (Betarbet et al., 2002; Cicchetti et al., 2009). However, the complexity of the pathology makes it difficult to reproduce the exact human diseases in animals, which results in unrealistic neuroprotection in animal models that cannot be extrapolated later to human patients (Le et al., 2014). Many neurotoxic chemicals and genetic models are used in research to mimic PD in animals for testing neuroprotective and neurorestorative therapies. Some of the commonly employed toxins are MPTP, rotenone and 6-OHDA (Bezard et al., 2013; Blesa and Przedborski, 2014).

Both the MPTP and rotenone models have their respective pros and cons and either/both should be selected according to the aims of the research (Betarbet et al., 2002). Moreover, different strains of mice show different sensitivity towards MPTP toxicity. For example, C57BL/6 mice show higher sensitivity than the BALB/c or Swiss Webster mice (Boyd et al., 2007; Hamre et al., 1999). MPTP generally is utilized in acute and subchronic models (Le et al., 2014) whereas rotenone is mostly used in chronic models probably because high doses cause acute toxicity resulting in high mortality rates (Meurers et al., 2009). The most commonly used dose of MPTP for a subacute regimen is 30 mg/kg/day. The LD50 of an intraperitoneal injection of rotenone in mice is 2.8 mg/kg (Gupta, 2012). Hence, we performed this study to compare the subchronic models of MPTP and rotenone using 30 mg/kg/day and 2.5 mg/kg/day, respectively.

We observed clear differences between the effects of the neurotoxins. Firstly, neurochemical analyses showed that the DA and DOPAC levels in the striatum were significantly decreased by MPTP, but rotenone caused minor to almost no changes compared to the control group (Fig. 1A and B). According to Thiffault et al. (2000), mice treated with 2.5 mg/kg of rotenone thrice weekly for three consecutive weeks manifested only a slight change in striatal DA and DOPAC (Thiffault et al., 2000), which is consistent with the results of the present study. Similarly, it was found that treatment with rotenone for the chronic model causes enhancement of the turnover of DA and its metabolites rather than suppression. (Richter et al., 2007).

The HPLC results were supported by TH immunohistochemical staining, in which MPTP caused a greater loss of dopaminergic neurons and fibers in the substantia nigra and striatum, respectively, while rotenone did not show any neurodegenerative effect in these regions. The behavioral studies correlated with these findings and indicated that MPTP induced reduction in locomotor activity compared to the saline and rotenone treated groups. Rotenone did not damage the nigrostriatal region and produced only a slight reduction in the activity of the animals (Fig. 1C). In a previous study, Richter et al. (2007) observed motor dysfunction with rotenone but the motor abnormalities were considered to result from mitochondrial complex I inhibition and peripheral toxicity rather than the degeneration of dopaminergic neurons (Richter et al., 2007). Thus, it is speculated that the rotenone-induced behavioral impairment might come from non-specific peripheral toxicity, but not from dopaminergic neurodegeneration in the CNS.

Regarding the glial activation, rotenone caused a significant activation of glial cells in the hippocampus only, whereas MPTP mediated glial activation in the nigrostriatal pathway but not in the hippocampus (Figs. 3 and 4). Astrocytes express and release a series of growth factors, including neurotrophic factors, which remodel the impaired

nigrostriatal milieu and promote the endogenous protective mechanism (Bélanger and Magistretti, 2009; Blackburn et al., 2009; L'Episcopo et al., 2013; Liberto et al., 2004; Voutilainen et al., 2009). The hippocampal region expresses high levels of BDNF and its receptor TrkB (Webster et al., 2006). In the present study, rotenone-induced activation of astrocytes in the hippocampal region suggests that this region is more susceptible to the toxin. This was confirmed by increased staining of the FJC in the hippocampal regions.

AMPK is a sensor for energy level, and the enzyme is activated by phosphorylation on the Thr172 residue of its alpha subunit when ATP level is too low. We observed pAMPK level was significantly increased after rotenone injection, which might be a compensatory response of the brain to the toxin. However, the results should be interpreted with caution because pAMPK level could be different depending on methods of killing and tissue preparation (Scharf et al., 2008). This is also the case of Akt. Therefore, increase in phosphorylation level of AMPK or Akt does not accurately reflect biological alterations.

Taken together, our results indicate that for the subchronic model, MPTP but not rotenone possesses the ability to produce neurodegeneration in C57BL/6 mice by causing a clear loss of dopaminergic neurons and fibers in the nigrostriatal pathway, promoting behavioral deficits and activation of glial cells. Rotenone on the other hand, neither caused atrophy of dopaminergic neurons nor produced significant behavioral impairment. More importantly, rotenone not only increased the BDNF and GDNF levels, but also activated their downstream signaling molecules along with AMPK. Thus, we might speculate that low doses of rotenone might be involved in the activation of a protective mechanism against its own neurotoxic effects, which prevents significant detrimental effects in the nigrostriatal pathway.

Conflict of interest

The authors have no competing interests to declare.

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