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Article

The neuroprotective effect of GPR4 inhibition through attenuation of caspase mediated apoptotic cell death in MPTP induced mouse model of Parkinson's disease

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Abstract: GPR4, a member of proton activated GPCRs group. Previously we have reported that GPR4 is constitutively active at physiological pH and knockout of GPR4 has shown to protect dopaminergic neuronal cells from caspase-dependent mitochondrial apoptotic cell death. In this study we have investigated the role of GPR4 in 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) treated mice model of Parkinson's disease. Subchronic administration of MPTP in mice produces oxidative stress induced apoptotic cell death of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and motor deficits. Treatment with NE52-QQ57, a selective antagonist of GPR4 reduced dopaminergic neuronal loss MPTP-intoxicated C57BL6/J mice and improved motor deficit and memory impairment. Co-treatment with NE52-QQ57 significantly decreases the protein level of the proapoptotic marker (Bax), and increases the antiapoptotic marker (Bcl-2) in the SNpc and striatum tissue collected from the brain of MPTP inflicted mice. Further, MPTP-induced activation of caspase 3 and cleavage of poly (ADP-ribose) polymerase (PARP) was significantly decreased in the SNpc and striatum tissue of NE52-QQ57 cotreated mice. Further mice receiving both MPTP and NE52-QQ57 mice showed significantly higher TH positive cells in the SNpc and striatum than MPTP treated mice alone. Moreover, NE52-QQ57 cotreatment improved the motor activity in the rotarod test and pole test and also improved spatial memory in Y maze test. Our findings suggest GPR4 as a potential therapeutic target for PD whereas the activation GPR4 is involved in the caspase mediated apoptotic cell death in SNpc and striatum of MPTP-intoxicated mice.

Keywords: Apoptosis, PARP, Caspase 3, Neurodegeneration, GPR4 receptor, MPTP, Parkinson's disease.

1. Introduction

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Parkinson's disease (PD), is a serious movement disorder and a second most progressive neurodegenerative disorder that affects people aged more than 60 years [1]. Its most prominent clinical manifestations include difficulty in coordinated movement such as bradykinesia, asymmetric resting tremor and rigidity [2]. The disease is attributed to a pathophysiologic loss or degradation of dopaminergic neurons in the substantia nigra pars compacta (SNpc) of the brain [3]. An array of evidence suggests that the major cause of dopaminergic neuronal loss in substantia nigra is reactive oxygen species (ROS) induced oxidative stress [4]. A number of sources suggest that mitochondrial dysfunction can lead to exacerbation of ROS generation and susceptibility to oxidative stress mediated dopaminergic cell death in brain [5].

A 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a dopaminergic pyridine toxin widely used neurotoxin mimic PD. To create a toxin-based PD animal model, MPTP is a gold standard which produces almost all of the human PD like pathological hallmarks. While injected in specific strains of mice like C57BL/6 MPTP induces the behavioral and systemic symptoms of PD patients such as severe motor deficits associated bradykinesia, resting tremors, rigidity, and postural instability, along with dopaminergic cell death in the SNpc and striatum of the brain due to oxidative stress mediated apoptosis [6, 7]. MPTP is metabolized in the glial cells of brain and converted to its active form 1-methyl-4-phenylpyridinium (MPP^+). In dopaminergic neurons MPP^+, is absorbed through solute carrier family 6 [neurotransmitter transporter], member 3 (SLC6A3/DAT) and disrupts the respiratory enzymes in mitochondria, causes oxidative damage [8, 9]. MPP^+ has been reported to generate more that 5-7 times reactive oxygen species (ROS). MPP^+ selectively inhibits the mitochondrial complex I and initiates the classical mitochondrial apoptotic pathway through a series of sequential events; opening the mitochondrial permeability transition pore which causes the collapse of mitochondrial membrane potential ($\Delta\Psi m$), imbalance of proapoptotic Bax/Bcl-2 ratio followed by the release of cytochrome c, activation of caspases-3, and proteolytic degradation of poly (ADP-ribose) polymerase (PARP) and subsequent apoptosis of the neuronal cells [13, 14][7].

G-protein coupled receptor 4 (GPR4), a proton-sensing receptor is a member of small family of G protein-coupled proton-sensing receptors consists of three other GPCRs namely; ovarian cancer G protein-coupled receptor 1 (OGR1), G2A and T-cell death-associated gene 8 (TDAG8), they are also known asGPR68, GPR132 and GPR65 respectively. GPR4 is a Gs, G_{13} and $Gq/_{11}$ coupled receptor which also modulate signals through cAMP. In several studies, GPR4 has been found to be activated between a range physiological pH (7.0-7.4) with little increase in cAMP if pH is slightly acidic [10]. Whereas, in the brainstem, pH is rather tightly balanced and kept within ~7.6-7.0. In our previous studies we have shown that GPR4 is activated in physiological pH (~7.4) in dopaminergic neuronal cells. Moreover, (Hosford et al., 2018) reported that GPR4 is expressed by multiple neuronal populations and endothelium of both mice and rat and suggested that the pH sensitivity of GPR4 is affected by level of expression in the cell type [11]. Transcriptome study shows that GPR4 is highly expressed in the peripheral endothelial cells (blood vessel formation) [12] and kidney (control of acid-base balance)[13]. Expression of GPR4 in some parts of brain brainstem especially retrotrapezoid nucleus (RTN), C1 catecholaminergic neurons and in serotonergic raphe nucleus neurons has been reported in many studies. Which also suggest the role of GPR4 in RTN in central chemosensitivity to CO2 [14]. Besides, GPR4 plays critical role in different biological functions such as inflammation, proliferation, paracellular gap formation of endothelial cells, apoptosis, growth, of cancer cells,

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angiogenesis etc. [15-17]. Previously, pharmacological inhibition of GPR4 has been reported to remediate myocardial infarction [18] and genetic deletion of GPR4 improved cardiac function through lowering blood pressure [19], and inhibited apoptosis in renal ischemia reperfusion injury [20] intestinal inflammation [21]. However, there was no study on the role of GPR4 on the apoptosis cell death neurodegenerative disease before we have investigated the effect of GPR4 knockout and overexpression in the dopaminergic neuronal cells [22]. Our previous study showed, GPR4 knockout or pharmacological inhibition improves the neurotoxin induced caspase 3 dependent apoptotic cell death. Here in this study, we have used sub chronically administered MPTP-induced Parkinsonism in C57BL/6J mice to further elaborate the role of GPR4 in the classical mitochondrial apoptotic cell death. We have used orally active GPR4 selective antagonist to evaluate how pharmacological inhibition of GPR4 protects neural cell loss in the SNpc through increasing the level of tyrosine hydroxylase (TH) and attenuation of caspase mediated apoptotic cell death.

2. Results

2.1. Expression of GPR4 is upregulated in MPTP-induced PD mouse model

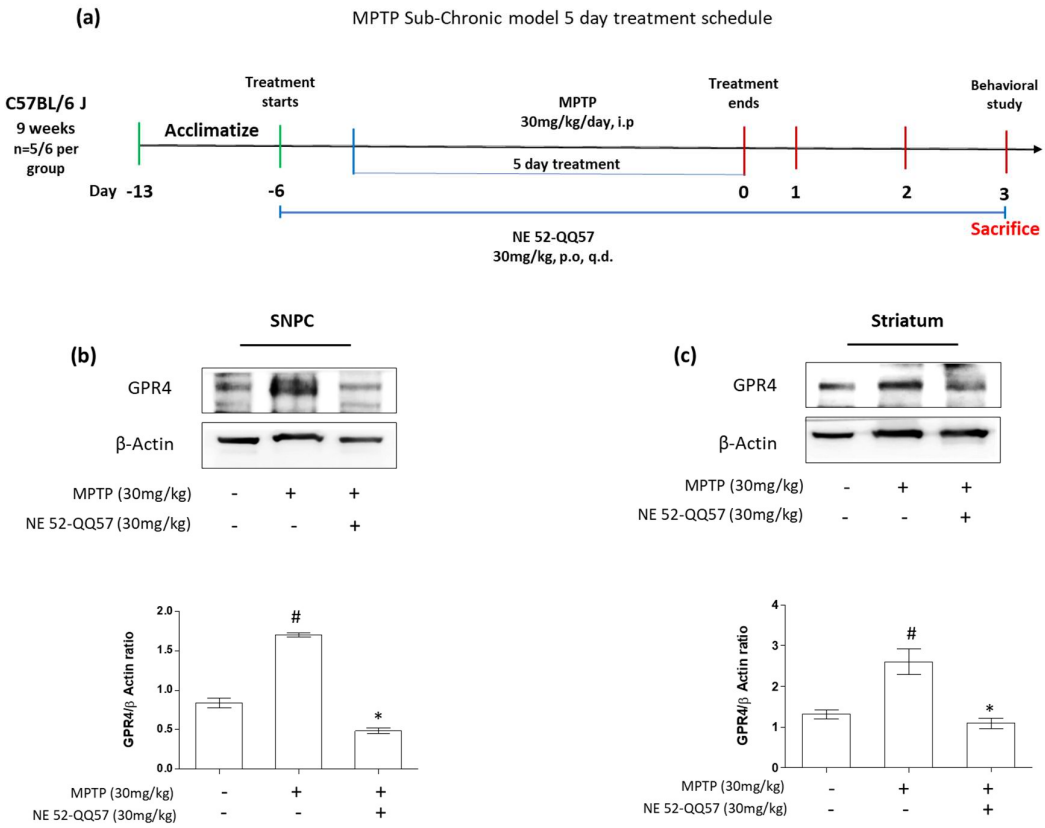

Figure 1: The effect of subchronic administration MPTP, on the GPR4 protein expression the Substantia nigra and Striatum of mice brain. (a) Representation of the treatment and behavioral study schedule for animal experiments. GPR4 protein expression and its densitometric analysis in (b). SNpc and (c). Striatum tissue collected after 3 day of last MPTP administration. β Actin ware used as an internal control. Data represented as mean ± SEM. Tukey's multiple comparison test of One-way ANOVA-was used. #$p < 0.05$ when the MPTP treated group is compared with the control group; *$p < 0.05$ other treated groups compared with the MPTP treated group.

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The dose of NE52-QQ57 (30 mg/kg/, p.o.) was adopted from Hosford et al., 2018 and MPTP (30 mg/kg, i.p.) for 5 days has been adopted from Kim *et al.*, 2015 [11, 23]. Mice were cotreated with NE52-QQ57 (30 mg/kg/, p.o.) and MPTP (30 mg/kg, i.p.) for 5 days but the NE52-QQ57 (30 mg/kg/, p.o.) treatment was continued till the day before sacrifice. After three days of last MPTP injection mice were sacrificed after behavioral studies and tissue of substantia nigra and striatum of mice brain were collected (Figure 1a).

It is acknowledged that the activation of GPR4 differs based on the cell types of the and number of GPR4 expressed in that cell. Different regions of brains have very distinct type of cell types which shows different level of GPR4 activities [11]. We have investigated the expression of the GPR4 receptor different regions of mice brain using immunoblot analysis (supplementary figure 1). We have found that the expression level of GPR4 in SNpc and striatum after subchronic administration of MPTP (30 mg/kg, i.p.) for 5 days was highest after the three day of last MPTP injection.

Subchronic administration of MPTP significantly ($p < 0.05$) increased the GPR4 protein level up to (1.69±0.2 fold) in SNpc (Figure 1b). Whereas, NE52-QQ57 treatment significantly ($p < 0.05$) decreased GPR4 protein level (0.48±0.04 fold) in the SNpc of NE52-QQ57 -MPTP cotreated group (Figure 1b). In striatum, GPR4 protein level was significantly ($p < 0.05$) increased more than two and a half fold (2.60±0.35) in MPTP treated group (Figure 1c). Whereas, NE52-QQ57 treatment significantly ($p < 0.05$) decreased the GPR4 protein level (1.09±0.14 fold) in the striatum of the NE52-QQ57-MPTP cotreated group (Figure 1c). These data suggest that MPTP administration has a correlation with the expression level of GPR4 in the SNpc and stritum of mice model of PD.

2.2. Inhibition of GPR4 protects from the MPTP-induced depletion of tyrosine hydroxylase in the SNpc and striatum

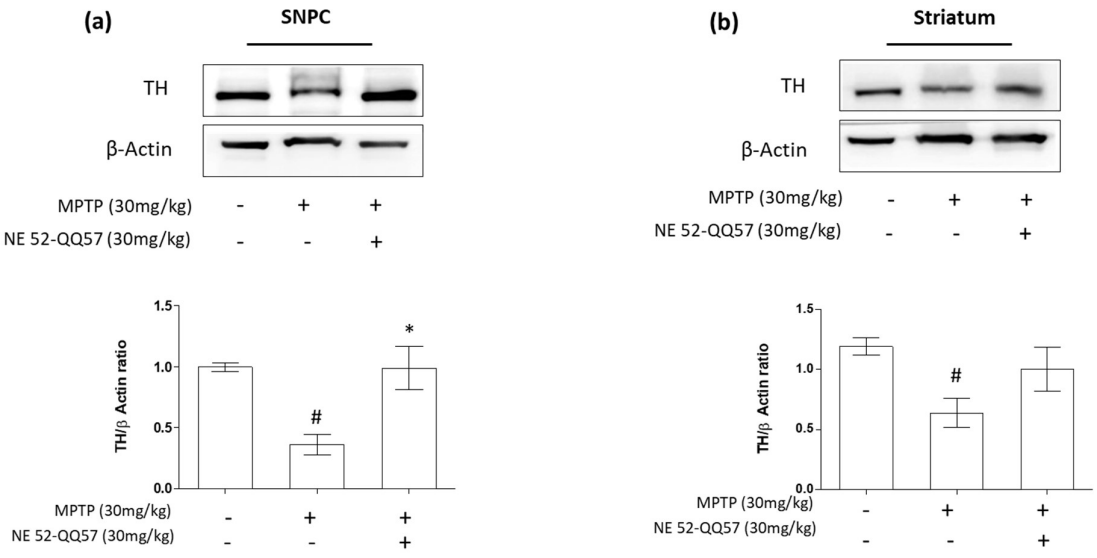

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Figure 2: The effect of GPR4 antagonist, NE52-QQ57, on the TH protein expressions in MPTP-treated mice. MPTP was treated (30mg/kg/day) for 5 days. Mice were sacrificed and substantia nigra and striatum tissue were collected after three days of last MPTP administration. (a). TH protein expression SNpc region of mice brain (n=3) and densitometric analysis. (b). TH protein expression striatum region of mice brain (n=3) and densitometric analysis. β-Actin was utilized as an internal control. Data represented as mean ± SEM. Tukey's multiple comparison test of One-way ANOVA-was used. #$p < 0.05$ when the MPTP treated group is compared with the control group; *$p < 0.05$ other treated groups compared with the MPTP treated group.

Treatment with MPTP (30 mg/kg, i.p.) for 5 days significantly ($p < 0.05$) reduced the TH-immunopositively to almost half (0.36±0.09 fold) ($p < 0.05$) in SNpc (Figure 2a). Whereas, NE52-QQ57 treatment significantly ($p < 0.05$) prevented the TH depletion (0.98±0.2 fold) in SNpc of the NE52-QQ57 -MPTP cotreated group (Figure 2a). In striatum, MPTP (30 mg/kg, i.p.) treatment for 5 days significantly ($p < 0.05$) reduced the TH-immunopositively to more than half (0.64±0.14 fold). Whereas, NE52-QQ57 treatment significantly ($p < 0.05$) and prevented the TH depletion (1.0±0.21 fold) in the striatum of the NE52-QQ57-MPTP cotreated group (Figure 2b). These data suggest that the depleted of TH protein by the subchronic administration of MPTP can be prevented by the inhibition of GPR4 receptor.

2.3. Inhibition of GPR4 decreases MPTP-induced increase of proapoptotic Bax/Bcl-2 ratio in SNpc and striatum

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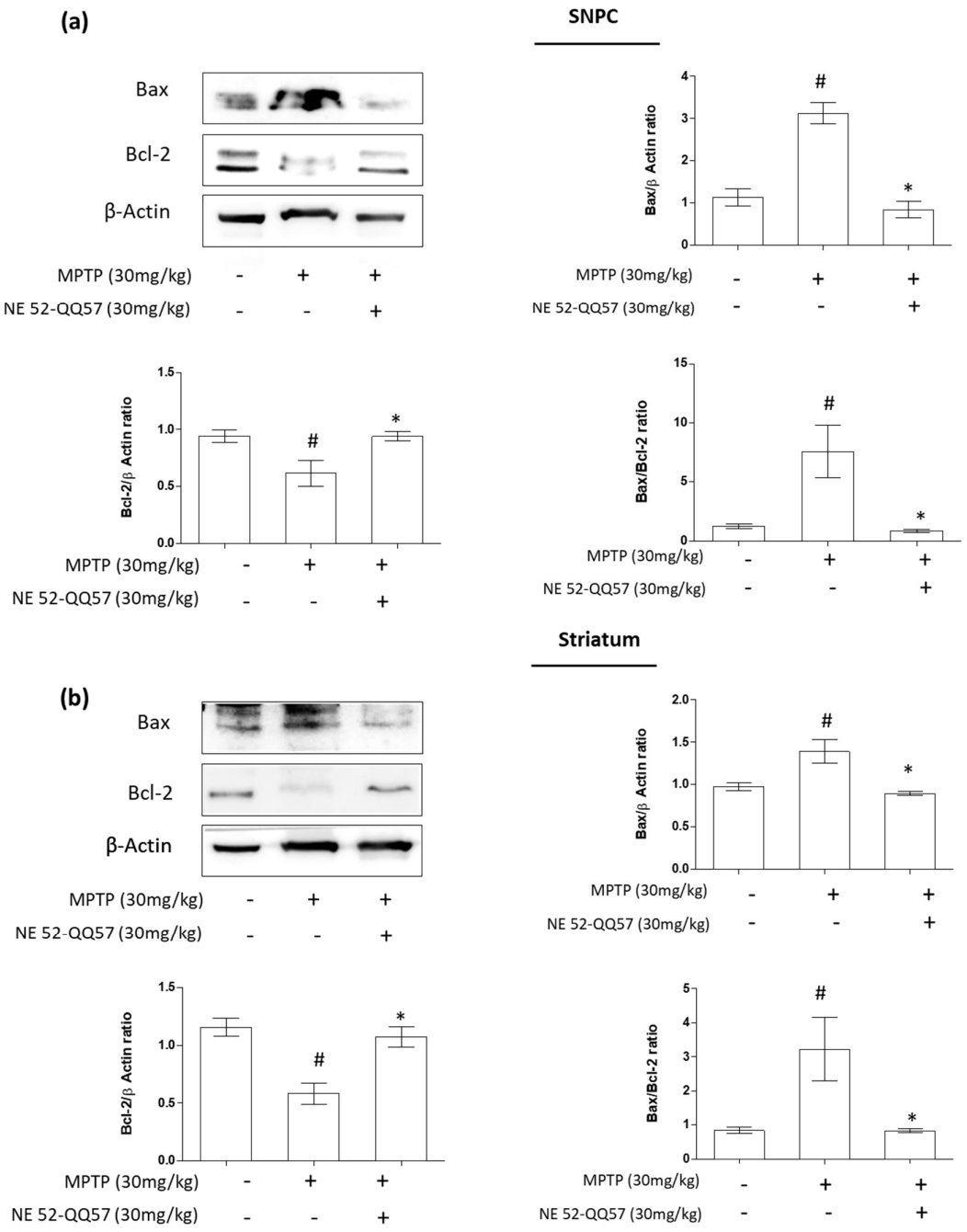

Figure 3: The effect of GPR4 antagonist, NE52-QQ57, on the pro-apoptotic Bax and Bcl-2 protein expressions in MPTP-treated mice. MPTP was treated (30mg/kg/day) for 5 days. Mice were sacrificed and Substantia nigra and Striatum tissue was collected after three days of last MPTP administration. (a). Bax and Bcl-2 protein expression SNPc region of mice brain (n=3) and densitometric analysis. (b). Bax and Bcl-2 protein expression Striatum region of mice brain (n=3) and densitometric analysis. β-Actin was utilised as an internal control. Data represented as mean ± SEM. Tukey's multiple comparison test of One-way ANOVA-was used. #$p < 0.05$ when the MPTP treated group is compared with the control group; *$p < 0.05$ other treated groups compared with the MPTP treated group.

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The Bcl-2 family proteins Bax and Bcl-2 play a role of central regulator of mitochondrial apoptotic pathway. Bcl-2 protein's activity is dependent on its counteracting twin Bax. Activation of BAX result is an initiation step of apoptosis, while Bcl-2 protects from the program cell death and promote cells survival. Activation of Bax initiates the release of cytochrome C from mitochondrial intermembrane space to the cytosol which activates the proteolytic caspases [24]. To investigate the effect of GPR4 inhibition on Bax and Bcl-2 protein expression mice were cotreated with NE52-QQ57 (30 mg/kg/, p.o.) and MPTP (30 mg/kg, i.p.) for 5 days and continued till the day before sacrifice. Three day after the last MPTP injection brain tissue from SNpc and striatum were collected and processed for immunoblot.

The level of Bax protein expression in SNpc of mice treated with MPTP led to a significantly ($p < 0.05$) increase, more than three folds (3.13±0.28 fold) and reduced the Bcl-2 protein expression to almost half (0.57±0.15 fold) ($p < 0.05$) comparison with vehicle only group (Figure 3a). The ratio of Bax/Bcl-2 shows more than seven-fold increase (7.55±2.57 fold) than the vehicle only group. Additionally, cotreatment of NE52-QQ57 with MPTP significantly ($p < 0.05$) prevented the increase of Bax expression (0.84±0.22 fold) and the level of Bcl-2 expression (0.95±0.05 fold) in the SNpc in comparison with MPTP treated group (Figure 3a). The Bax/Bcl-2 ratio in the SNpc of NE52-QQ57 and MPTP co treated group was also significantly ($p < 0.05$) lower (0.86±0.17 fold) than the MPTP treated group.

In the tissue collected from striatum of MPTP treated group of mice, the expression level of Bax was significantly ($p < 0.05$) increased (1.39±0.16 fold) and the level of Bcl-2 expression was almost half (0.52±0.09 fold) fold than the vehicle treated group. Whereas, the Bax/Bcl-2 ratio was significantly ($p < 0.05$) higher (3.22±0.82 fold) than the vehicle treated group (Figure 3b). However, in NE52-QQ57 -MPTP cotreated group significant ($p < 0.05$) decrease of the Bax expression (0.89±0.024 fold) and less depletion of Bcl-2 protein expression (1.11±0.11 fold) was observed than the MPTP treated group. Moreover, the Bax/Bcl-2 ratio in NE52-QQ57 -MPTP cotreated group was also significantly ($p < 0.05$) lower (0.82±0.06 fold) than the MPTP treated group (Figure 3a). These data suggest that sub chronic administration of MPTP causes the imbalance of mitochondrial Bax/Bcl-2 protein ratio and that may initiate the mitochondrial caspase mediated apoptotic pathway. Treatment with selective GPR4 antagonist NE52-QQ57 prevents the increase of proapoptotic protein Bax and also prevent the depletion of antiapoptotic protein Bcl-2 which indicates the anti-apoptotic effect of NE52-QQ57.

2.4. Effect of GPR4 inhibition on the cleavage of PARP and caspase 3 activity in the SNpc and striatum of MPTP-induced PD mouse model

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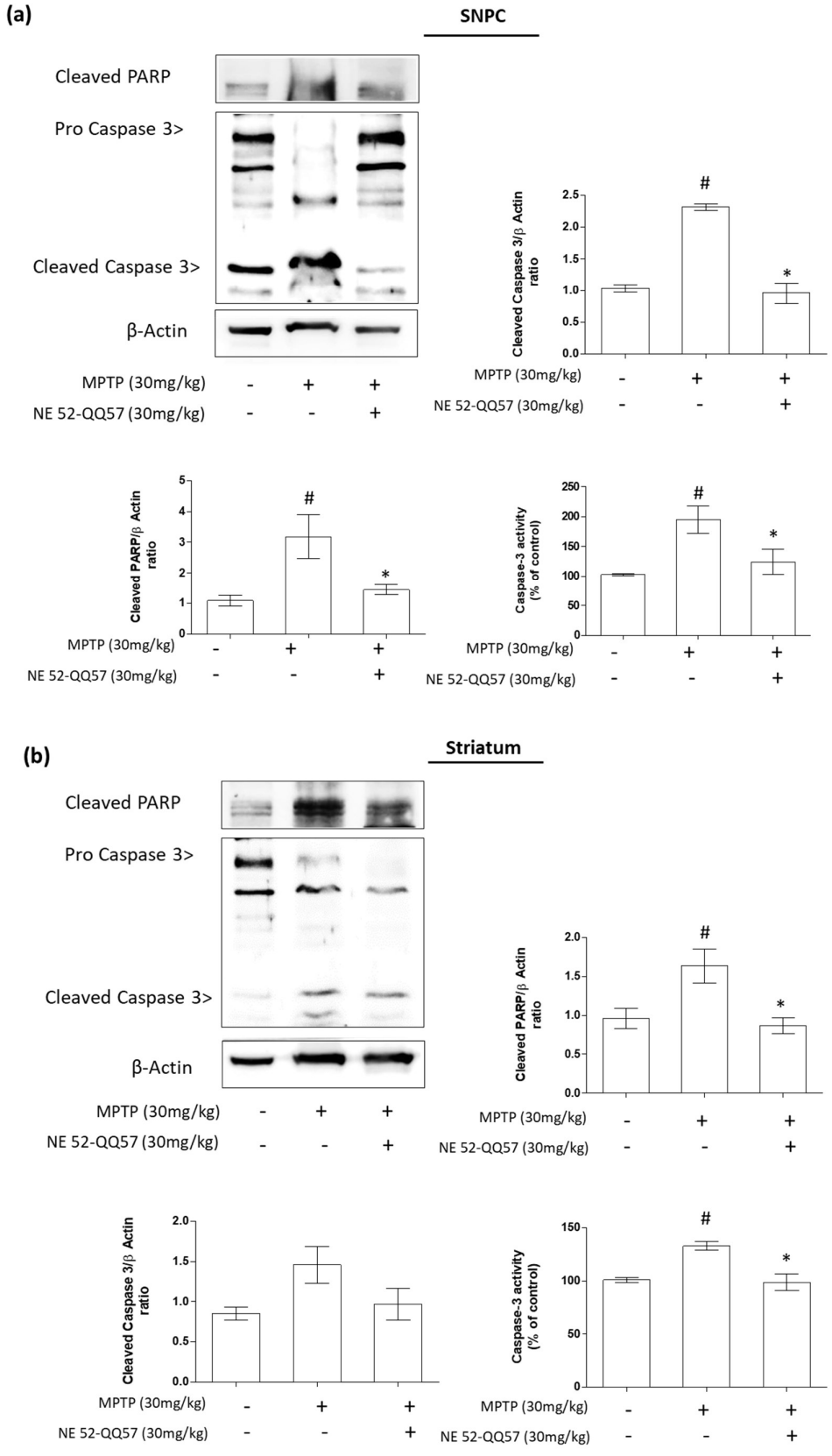

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Figure 4: The effect of GPR4 antagonist, NE52-QQ57, on the expression of pro-apoptotic proteins cleaved PARP, cleaved Caspase 3 and caspase 3 activity in MPTP-treated mice. MPTP was treated (30mg/kg/day) for 5 days. Mice were sacrificed and substantia nigra and striatum tissue was collected after three days of last MPTP administration. (a). Cleaved PARP-1 and Cleaved Caspase 3 protein expression and Caspase 3 activity assay of SNPc tissue region of mice brain (n=3) and densitometric analysis. (b). Cleaved PARP-1 and Cleaved Caspase 3 protein expression and Caspase 3 activity assay of striatum region of mice brain (n=3) and densitometric analysis. β-Actin was utilized as an internal control. Data represented as mean ± SEM. Tukey's multiple comparison test of One-way ANOVA-was used. #$p < 0.05$ when the MPTP treated group is compared with the control group; *$p < 0.05$ other treated groups compared with the MPTP treated group.

In response to oxidative stress and poly(ADP-ribose) polymerase (PARP), is the most abundant nuclear enzyme. The cleavage of PARP is a reliable marker of apoptosis which is done by DEVD-ase caspases, a family of proteases like caspase 3 activated during apoptosis [25, 26]. To further access the effect of GPR4 inhibition by NE52-QQ57 on apoptosis of dopaminergic neurons, we have measured the level of cleaved PARP, its proteolytic enzyme caspase 3 protein and caspase activity of SNpc and striatum tissue collected from MPTP inflicted mice.

After treatment with MPTP (30 mg/kg, i.p.) for 5 days the level of cleaved PARP protein expression in SNpc of mice led to a significant ($p < 0.05$) increase of cleaved PARP (3.78±0.6 fold) and cleaved caspase 3 protein to almost two and a half (2.32±0.06 fold) ($p < 0.05$) comparison with vehicle only group (Figure 4a). Additionally, in a separate quantitative caspase 3 activity assay MPTP treated group showed more than double caspase 3 activity (194.923±25.86%) than the vehicle treated group. Whereas, cotreatment with NE52-QQ57 and MPTP prevented the increase of cleaved PARP (1.56±0.14 fold) and cleaved Caspase 3 (0.96±0.19 fold) and also prevented the increase of caspase 3 activity (123.847±23.63%) than the MPTP treated group alone.

However, in the striatum MPTP treated mice group significantly (#$p < 0.05$) increased cleaved PARP (1.84±0.11 fold) and cleaved caspase 3 level (1.46±0.26 fold). Also, the quantitative analysis of caspase 3 activity showed a significant (#$p < 0.05$) increase of (133.03±4.87%) caspase 3 activity in the MPTP treated mice. Whereas, cotreatment with NE52-QQ57 and MPTP prevents the increase of cleaved PARP (0.85±0.14 fold), cleaved caspase 3 level (0.97±0.23 fold) and significantly (*$p < 0.05$) prevented the increase of caspase 3 activity (98.87±9.02%) (Figure 4b). Overall, the data suggest that inhibition of GPR4 has a strong effect on the inhibition of apoptotic cell deaths through inhibition of mitochondrial caspase 3 mediated cleavage of PARP. However, of GPR4 inhibition prevented the MPTP mediated increase of caspase 3 activity in both SNpc and striatum tissues but the protective effect of NE52-QQ57 was higher in SNpc than the striatum.

2.5. Effect of GPR4 antagonist on the MPTP induced degradation of TH-positive cells in SNpc and striatum.

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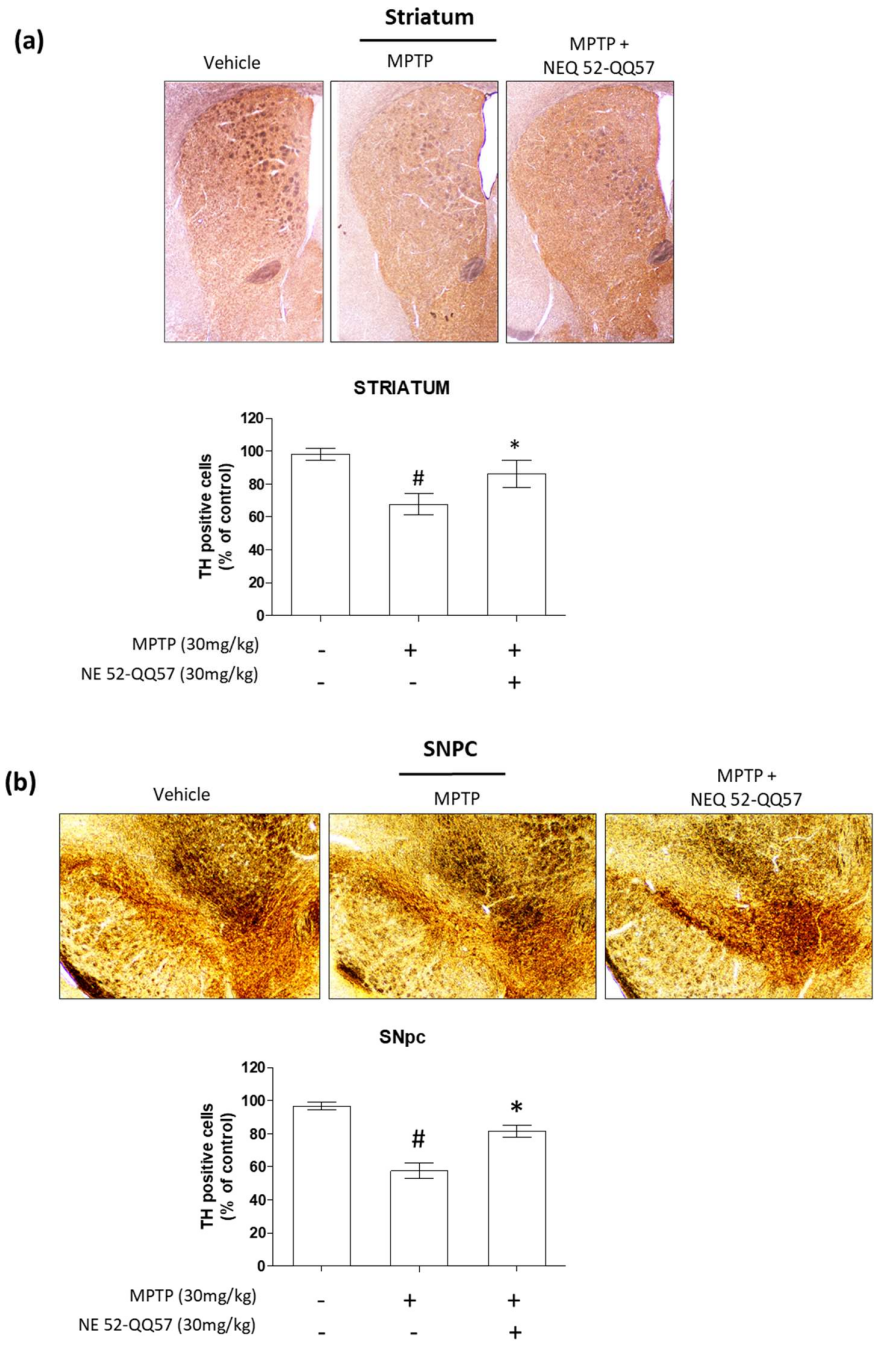

Figure 5: GPR4 inhibition attenuates the depletion of TH positive cells in the striatum and SNpc of MPTP-intoxicated sub-chronic mice model of PD. MPTP was treated (30mg/kg/day) for 5 days. After performing the behavioral experiment mice were anesthetized for the immunohistochemical study three days after the last MPTP administration. Mice were sacrificed and whole brain was isolated to collect sections of SNpc and Striatum region. (a). Representative images of TH-positive cell immunoreactivity (IR) in striatum sections with densitometric analysis for TH protein expression (n = 3-4). (b). Representative images of TH-positive cell immunoreactivity (IR) in SNpc sections with densitometric analysis for TH protein expression (n = 3-4). Data represented as mean ± SEM. Tukey's multiple comparison test of One-way ANOVA-was

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used. #p < 0.05 when the MPTP treated group is compared with the control group; *p < 0.05 other treated groups compared with the MPTP treated group.

Tyrosine hydroxylase (TH), an enzyme that converts L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA). The conversion of L-tyrosine to DOPA is the rate-limiting and initial step in the dopamine biosynthesis pathway in dopaminergic neurons [27]. Moreover, TH is widely used as a marker for dopaminergic neurons in the CNS. To evaluate the effect of NE52-QQ57 on TH positive cells of in SNpc and striatum mice were intoxicated with MPTP (30 mg/kg, i.p.) and cotreated with NE52-QQ57 (30 mg/kg/, p.o.) and for 5 days. NE52-QQ57 treatment was continued till the day before sacrifice. Three day after the last MPTP injection mice were deeply anesthetized followed by transcardial transfusion and fixation brains were isolated. Immunohistochemistry was performed to measure reactivity of TH positive neurons in sections of SNpc and striatum.

In the striatum a significant reduction of TH-positive cells was observed after subchronic administration of MPTP. Whereas cotreatment with NE52-QQ52 protected from the loss of TH positive cells in NE52-QQ52-MPTP cotreated group (Figure 5a). Densitometric analysis of the image shows MPTP treatment significantly (#p < 0.05) lowered the TH positive cells to almost half (87.07±1.53%) in comparison with vehicle treated group. Whereas, NE52-QQ52 cotreated group significantly (*p < 0.05) prevented the loss of TH positive cells (103.73±2.56%).

In SNpc tissues similar phenomenon was observed as MPTP significantly reduced the number of TH-positive cells and MPTP-NE52-QQ57 group showed lower depletion of TH positive cells (Figure 5b). Quantitive representation of images shows significant depletion of Th positive cells (62.46±6.56%) in comparison with vehicle treated group. Whereas in the MPTP and NE 52-QQ57 cotreated group showed significant (*p < 0.05) protection against the depletion of TH positive cell (101.74±6.53%). Although, in both SNpc and striatum MPTP significantly damaged the TH positive dopaminergic neurons the amount of depletion in SNpc is higher than the striatum. Overall, the data suggest that NE52-QQ57 strongly protected the loss of TH positive cells in both major location of TH positive cell depletion in the brain of MPTP treated mice.

2.6. Effect of GPR4 antagonist on the motor coordination function (Rotarod), bradykinesia (pole test) and spontaneous alternation performance (Y maze test) on MPTP induced PD mouse model

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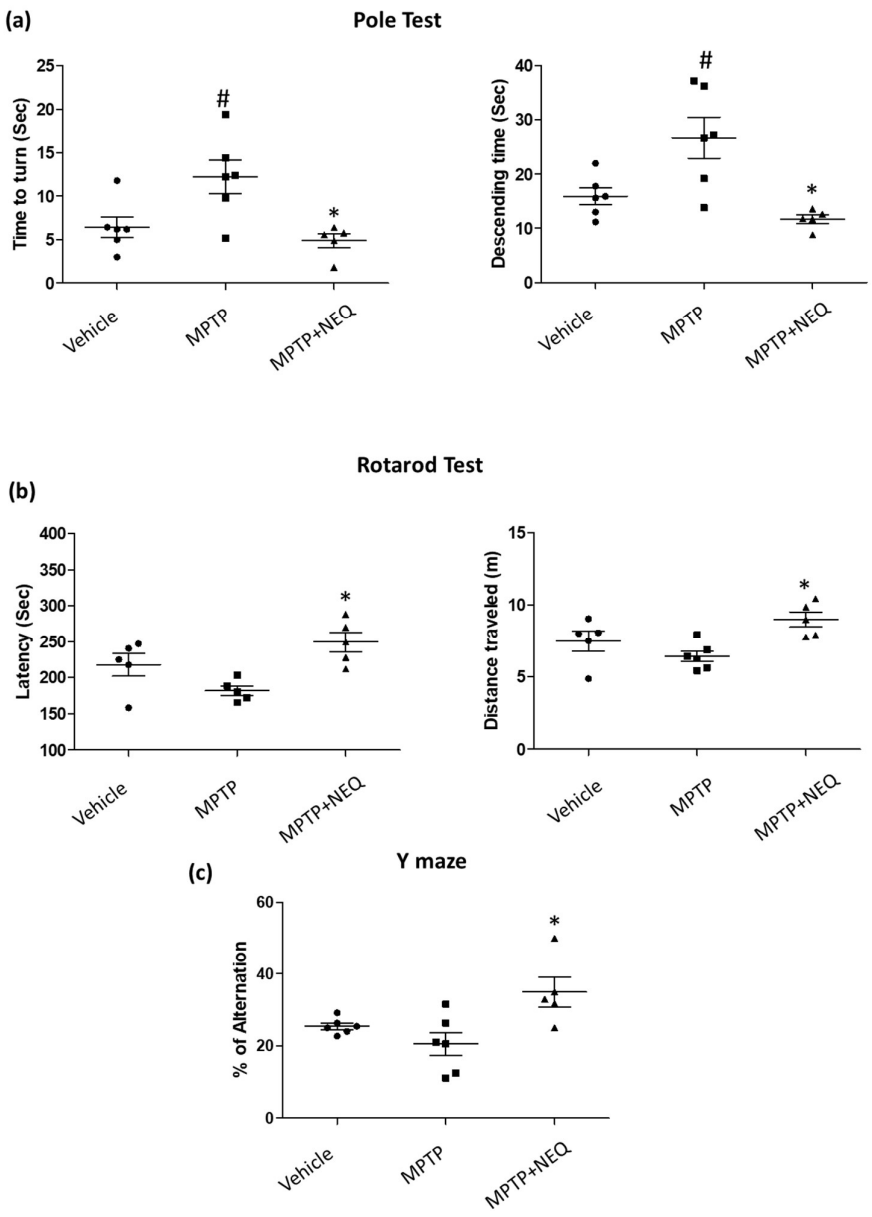

Figure 6: GPR4 inhibition attenuates motor deficits in MPTP-intoxicated sub-chronic mice model of PD. MPTP was treated (30mg/kg/day) for 5 days. Behavioral experiment were aperformed three days after the last MPTP administration (a). PD-like bradykinesia was measured using a pole test. (b). Motor coordination function was measured using a Rotarod. Latency period and distance travelled was measured. (c). Spatial memory measured using Y maze. Each dot represents the average of five individual trials and a thick bar is mean ± standard deviation. Data represented as mean ± SEM (n = 5 of triplicates). Data represented as mean ± SEM. Tukey's multiple comparison test of One-way ANOVA-was used. #$p < 0.05$ when the MPTP treated group is compared with the control group; *$p < 0.05$ other treated groups compared with the MPTP treated group.

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To investigate the effect of GPR4 inhibition on MPTP induced behavioral deficit we performed the pole test, rotarod and Y maze to evaluate the motor coordination function, bradykinesia and spontaneous alternation performance. Three day after the last MPTP treatment all the behavioral tests were performed. In pole test MPTP treated mice group showed significant increase (#$p < 0.05$) in the time to turn (12.24±1.92 sec) and descending time (26.72±3.75 sec). The latency to fall (188.62±7.87 sec) and distance travelled (6.434±0.34 m) were also decreased in the MPTP treated mice group in comparison with vehicle treated group. Additionally, MPTP treatment decreased the percentage of alteration (20.52±3.2%) in comparison with vehicle only group. On the other hand, NE52-QQ57-MPTP cotreatment shows significantly (*$p < 0.05$) less time to turn (4.9±0.66 sec) and descend (11.7±0.65 sec) than the MPTP only treated group (Figure 6a). NE52-QQ57-MPTP cotreatment treatment group also showed significantly (*$p < 0.05$) higher latency to fall (249.5±11.19 sec) and more distance traveled (9.01±0.43m) than the only MPTP treated group (Figure 6b). Moreover, in Y maze test the percentage of alteration (32.95±4.17%) was significantly (*$p < 0.05$) higher than the MPTP treated group alone (Figure 6c). Overall, these data suggest that GPR4 plays an important role in MPTP induced pathogenesis of PD. Thus, inhibition of GPR4 can improve the MPTP inflicted behavioral deficit in mice model of PD.

3. Discussion

In this study, we have investigated the effect of pharmacological inhibition of GPR4 in the mitochondrial oxidative stress-induced apoptotic cell death in PD. Activation of pH has been reported in many different pH ranges in different cells and animal model. Hosford and his team have also reported the expression of GPR4 in cerebrovascular endothelium and neurons of retro-trapezoidal nucleus locus coeruleus, dorsal raphe, and lateral septum of mice at physiological pH. We have shown the GPR4 is activate in the physiological pH (supplement data S1) and capable of potentiating the effect of MPP^+, an active metabolite of MPTP [22]. Here we intended to find out if MPTP treatment has any effect on GPR4 expression level over time in cerebellum, cerebral cortex, substantia nigra, hippocampus, pons medulla, mid brain and striatum of mice brain after 1, 3 and 7 days of last MPTP injection. In cerebellum and cerebral cortex no significant change in the expression of GPR4 were observed. Whereas, significant increased in the GPR4 level in the SNpc, hippocampus, midbrain and striatum after three days of post MPTP administration and slightly decreased in the seventh day was noticed (Supplementary figure 1). Thus, we have selected three days post MPTP treatment to collect SNpc and striatum tissues and behavioral studies. Interestingly in pons medulla GPR4 protein level was gradually increasing from day three to seven day of post MPTP administration. As suggested by Hosford *et al.*, 2018, the activity of GPR4 is related with the expression level of GPR4 in different tissues which justifies the variation of the level of GPR4 expression in different tissues is due to the level of expression of GPR4 at different population of neuronal cells and tissue in the brain.

SNpc, hippocampus, striatum, mid brain are the main regions of PD and probably the site where MPTP is converted to MPP^+ due to high number of glial cells and TH positive dopaminergic cells. Notably, our immunoblot and IHC results also suggest that the GPR4 expression may be connected with the apoptotic cell death of TH positive dopaminergic neurons in the midbrain, SNpc, striatum after three days of the administration of MPTP. Further to investigate the effect of GPR4 inhibition

on the neuronal loss, deficits in memory and motor performance Parkinson's disease model we have used selective GPR4 antagonist NE52-QQ57 on MPTP induced Parkinson's disease model. TH, an essential enzyme in dopamine biosynthesis converts tyrosine to dopamine by using tetrahydrobiopterin and molecular oxygen [27]. The decrease of TH activity is positively correlated with the dopaminergic neuronal cells. Moreover, behavioral deficit is also correlated with the number of TH-positive neurons in SNpc and striatum [23]. We found that GPR4 inhibition protects from the loss of TH in the striatum and SNpc in response to MPTP intoxication. Indicating that GPR4 inhibition may improve the dopaminergic loss associated motor activity and memory deficit.

To further investigate how selective GPR4 inhibition prevents the MPTP intoxicated neuronal loss through the mitochondrial oxidative stress mediated apoptotic pathway we investigated the ratio of Bax and Bcl-2 protein as early indication of the early phases of apoptotic cascade [28, 29]. MPTP inflicted apoptotic cell deaths bear the characteristic hallmarks of an increase in the Bax/Bcl-2 ratio, the release of cytochrome-C, and the Caspase-3 activation, which cleaves PARP and induces apoptotic cell death [30]. Previous studies suggest that MPTP administration up-regulates the expression of Bax and decreases the expression level of Bcl-2 in the SNpc and striatum of mice. These changes were found parallel with MPTP-inflicted dopaminergic neurodegeneration. Interestingly, studies also showed mice lacking Bax are resistant to MPTP [30, 31]. In our study GPR4 inhibition significantly prevented the increase of Bax and decrease of Bcl-2 protein thus restoring the Bax/Bcl-2 ratio in the SNpc and striatum region of the MPTP treated mice. Although in the SNpc tissue the ratio of Bax/Bcl-2 was higher than the striatum, the level of restoration of the Bax/Bcl-2 by NE52-QQ57 was almost same. Many studies suggested that the balance between the Bax/Bcl-2 ratio is an indicative of protection against apoptotic cell death [32-34]. The MPTP mediated mitochondrial oxidative stress results in the initiation of series of downstream events including Caspase activation and cleavage of PARP which consumes ATP, and thus acutely depletes cell energy stores of dopaminergic neurons. We further investigated the cleavage of caspase 3, caspase 3 activity and cleavage of PARP. Cleavage of PARP is a major substrate of caspases, which is also a valuable marker of apoptosis. In MPTP induced mitochondrial oxidative stress a dramatic increase of caspase 3 activity and PARP cleavage has been reported in neuronal cell death in brain, where caspase-3 has been reported to play as the final effector of caspase-dependent apoptosis [35, 36]. Our results show that MPTP dramatically increases the protein level of both cleaved caspase 3 and cleaved PARP in both SNpc and striatum. Additionally, the caspase 3 activity was also significantly increased in both SNpc and striatum tissues. Although the fold of cleaved caspase 3 and PARP proteins and caspase 3 activity was not same in both tissues, NE 52-QQ57 cotreatment significantly prevented the cleavage of both proteins and caspase3 activity in SNpc and striatum. Which demonstrate the pharmacological inhibition of GPR4 prevents the MPTP induced mitochondrial oxidative stress and prevent the caspase 3 dependent apoptotic cell death. This result is equivocal with our previously published *in vitro* data using both genetic and pharmacological inhibition of GPR4 in the human dopaminergic neuronal cell [22].

To further confirm the number dopaminergic cell loss is prevented by the GPR4 inhibition we investigate the immunoreactivity of TH positive cells through SNpc and striatum tissues through immunohistochemistry (IHC). Surprisingly the IHC of SNpc and striatum sections of the brain showed that GPR4 inhibition significantly prevented the depletion of TH positive cells in MPTP treated mice. Notably, this finding is equivocal with our immunoblot data of TH protein in SNpc and

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striatum. Moreover, in this study, the level of depletion of TH positive cells in the MPTP treated mice is similar to the level of TH positive cell depletion in the subchronic model of PD reported previously [37]. Our finding shows similarity with previously studies where demonstrating that TH activity is correlated with the behavioral deficit in toxin induced animal model of PD [23, 28].

Further, we investigate if the pharmacological inhibition of GPR4 has any effect on the memory impairment and behavioral deficit we performed, Rotarod, Y-maze and Pole tests. The pole test is used as a behavioral test to assess the bradykinesia in PD mouse models [38]. Pharmacological inhibition of GPR4 receptor improved the MPTP induced bradykinesia of mice. To evaluates the motor activity we have used the rotarod test which is commonly used in the mice model of PD [39]. The rotarod results show GPR4 antagonist significantly prevented MPTP induced motor deficit in mice. Cognitive impairment of spatial memory is reported after subchronic MPTP injection in different studies [40-42]. To assess the impairment of spatial memory we have used Y maze test which is widely performed in MPTP treated subchronic model of PD. Here our study shows that inhibition of GPR4 activity significantly improved the impaired spontaneous alteration in MPTP treated mice. Interestingly our immunoblot data suggest the higher level of GPR4 expression in the hippocampus region also suggest that GPR4 activity in may be connected with the memory deficit in neurodegenerative disorders like PD which provides a platform for further investigation of the involvement of GPR4 in memory deficit.

Taken together, our study suggests the subchronic administration of MPTP in mice induces classical mitochondrial oxidative stress mediated dopaminergic neuronal loss in the SNpc and the striatum. MPTP induced mitochondrial oxidative stress reduces the Bax/Bcl-2 ratio, increased caspase 3 activity mediated proteolytic degradation of PARP and subsequent apoptosis. Thus, the number of TH positive neurons is depleted in SNpc and the striatum over time. As a result, mice develop motor deficit and cognitive impairment which is restored by the pharmacological inhibitions of GPR4. In our previous study, we speculated that the GPR4 activation potentiates the neurotoxin mediated apoptotic cell death through PLC-β mediated release of intracellular calcium. Here we could not investigate such a phenomenon in animal model of PD. However, this study encourages further investigation of the cell-specific expression of GPR4 in SNpc and striatum and how GPR4 inhibition modulates the amelioration of apoptotic cell death in PD.

4. Materials and Methods

4.1. Reagents and Antibodies

NE 52QQ57 was purchased from MedChemExpress USA (Monmouth Junction, NJ, USA). 3-(3,4-dimehylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was brought from Sigma-Aldrich (St. Louis, MO, USA). RIPA buffer (10×) was purchased from Millipore (Milford, MA, USA). Tween 80 was purchased from Calbiochem (Gibbstown, NJ, USA). All other chemicals used in this research were of analytical grade and were purchased, unless otherwise noted, from Sigma-Aldrich.

4.2. Animals and MPTP and NE 52QQ57 Administration

Male C57BL6/J mice (age, 8–9 weeks; weight, 25–28 g) (Samtako Bio Korea, Gyeonggi-do, Korea) were acclimatized for 14 days, prior to the drug treatment. The Institutional Animal Care and Use Committee of Konkuk University approved all the Animal experiments and the experimental

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procedures(KU20207, 22/12/2020). The animals were housed in a controlled environment (23 ± 1° C and 50 ±5% humidity) and were allowed food and water ad libitum. The room's lights were on between 8:00 a.m. and 8:00 p.m. For the s/elective inhibition of GPR4 orally active GPR4 NE 52QQ57 has been used. The dose of NE 52QQ57 has been adjusted based on the previously published article by Hosford et al., /2018 [11].

Seventy-four mice were randomly divided into the following nine groups, comprised of 18 animals each: Vehicle group, MPTP (30 mg/kg/10 mL) group, and NE 52QQ57 (30 mg/kg/10 mL) MPTP (30 mg/kg/10 mL) group. As demonstrated in the experimental design, a 30 mg/kg/10 mL/day MPTP was administered intraperitoneally (i.p.) for five days to all of the animals except for those in the vehicle group. NE 52QQ57 (30 mg/kg/10 mL/day) i.e., per se group was orally administered one hour before MPTP injection, NE 52QQ57 was administered once daily, from one day prior of MPTP injection till one day before sacrifice. The animals were sacrificed after one day, three day and seven day of last MPTP injection. The MPTP solution in saline was freshly prepared before use. NE 52QQ57 was suspended in 10% DMSO, 0.5% methyl cellulose solution containing 0.5% Tween 80, before dosing. The plain methyl cellulose solution was administered as vehicle for control group.

4.3. Immunoblot analysis.

The immunoblot assays of target proteins were performed using our general lab protocol reported in previous publication. First mice brain tissues from different sections of brain (cerebellum, cerebral cortex, substantia nigra, hippocampus, pons medulla, midbrain and striatum), collected and cell lysates were prepared using the protein RIPA lysis buffer (Milford, MA, USA). The protein concentration of each tissue sample was measured using a DC Protein Assay kit (Bio-Rad). In 8%, 10% and 12% sodium dodecyl sulfate-polyacrylamide gels equal amounts of proteins (20–30 μg) were loaded. Proteins were electrophoretically separated and transferred to Nitrocellulose membranes (Millipore, Bedford, MA, USA). Each membranes individually incubated with corresponding primary antibodies GPR4 (1:500) (Novus Biologicals (Centennial, CO, USA)), Bax (1:1000) from Cell Signaling Co. (Boston, MA, USA), BCL-2 (1:1000), Caspase-3 (1:1000), Cleaved caspase-3 (1:1000), cleaved PARP (1:1000) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), PIP2 (1:500) from Abcam (Cambridge, United Kingdom) β-Actin (1:2000) from Sigma-Aldrich (St. Louis, MO, USA) overnight at 4 °C, followed by 1 h incubation with secondary antibodies conjugated with horseradish peroxidase (HRP) (1:2000; Cell signaling, MA). To visualize the bands blots were incubated with Biorad-ECL (Bio-Rad Laboratories) (Hercules, CA, USA) and photographed using ImageQuant™ LAS 500 (GE Healthcare) (Chicago, IL, USA). ImageJ (NIH) software was used to calculate the pixel intensity for each band. β-Actin was used to normalized the band intensity and to quantify the relative expression of other bands.

4.4. Assessment of Caspase-3 Activity

The Caspase-3 activity of brain tissue lysates were measured by Colorimetric Caspase-3 Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) described previously [x]. The 200 uL of reaction mixture containing 50 ul of protein was carried out in 96-well plate was incubated at 37°C for 180 min. The absorbance values reaction mixtures were measured at the wavelength of 405 nm in a microplate reader (Tecan Microplate Reader) (Meilen, Zurich, Switzerland).

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4.5. Immunohistochemistry

Mice were anesthetized with Chloroform after performing the behavioral experiments and transcardially perfused with phosphate buffered saline (PBS), and then by 4% paraformaldehyde (PFA) [6]. After perfusion fixation brains were removed and immersed in 4% PFA at 4◦C and dehydrated in 30% sucrose solution at 4◦C. Then they were embedded in tissue freezing medium (Leica, Gmbh Heidelberger, Germany). Frozen brans were cut in freezing microtome. Free floating sections (45 μM) of the striatum and SNpc regions were used for immunohistochemistry (described previously) [23]. Primary antibodies used for TH and was anti-TH rabbit antibody (1 : 1000; Millipore, Darmstadt, Germany). As secondary antibody biotinylated anti-rabbit (Vector Laboratories, Burlingame, CA, USA) was used for 1 h, then incubation in the Vectastain Elite avidin-biotin-peroxidase complex (ABC) Kit (Vector Laboratories, Darmstadt, Germany) for 60 min at room temperature as per manufacturer's recommendations. Finally, the sections were incubated in DAB substrate kit (Vector Laboratories) for color development, dried and mounted in glass slides. Images of stained sections were taken by a bright field microscope (Carl Zeiss Inc., Oberkochen, Germany). Image J software NIH, (Bethesda, MD, USA) is used to calculate the number of TH-positive cells in SNpc and striatum respectively. Data were represented as a percentage of the vehicle group values.

4.6. Behavioral studies

Rotarod test: Test was performed with slight modifications as described previously [31] After one day, three day and seven day of the last MPTP injection, rotarod performance was evaluated on the suspended rod (diameter: 3 cm) of an accelerating rotarod apparatus that accelerates at a constant rate from 1 to 40 rpm for 300 s. Mice were trained for 3 consecutive days, and they were placed on the rod for five trials. Time was auto recorded for each trial. Distance travelled before stopping or falling was also measured. A trial was considered ended when any mouse fell off the rotarod or when time reached 300 s. A 180 s of resting time was allowed between each trial.

Pole test: Pole test to investigate bradykinesia in mice was conducted based on our previous work with slight modification [23]. At the top of a rough surfaced wooden pole mice were placed upwardly which was 8 mm in diameter and 55 cm tall. The total time to descend and time to turn was measured. The time until the mouse arrived at the floor is referred as Time to descend. Delay or extension or of the time which usually takes to complete the test was considered as a reflection of bradykinesia. Each mouse was subjected to performed the test for five times successively.

Y-Maze test: To assess the spontaneous alternation performances of mice Y maze test was performed using a similar procedure described previously in [23]. Spontaneous alternation performances were assessed by recording animal behavior during a single session in a Y-maze. In the Y maze apparatus, each arm was 40 cm long, 12 cm wide and 30 cm in height. Each mouse being naïve or previously unintroduced to the maze, was placed at the end of one arm and allowed to move freely through every arm of the maze. During an 8 min session total entry to arms and spontaneous alteration of arms entries were recorded visually. Arm entry was considered to be accomplished when the hind paws of a mouse was completely placed in any arm. Whereas, alternation was considered when a mouse enters into all the three arms on overlapping triplet sets. The spontaneous alternation performances was calculated as percentage alternation based on the formula: percent alternation = [(number of alternations)/(total number of arm entries − 2)] × 100%.

4.6. Statistical Analyses

GraphPad Prism software version 5 (GraphPad, La Jolla, CA, USA) is used to perform statistical analyses. Data are represented as means ± standard error (SEM) of three to five independent experiments. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was performed to determine the significant differences between the nontreated and treated groups. A P-values < 0.05 were considered statistically significant.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/xxx/s1.

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Informed Consent Statement: Informed consent was obtained from the Institutional Animal Care and Use Committee of Konkuk University (KU20207, 22/12/2020) for all the animal experiments and the experimental procedures.

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Conflicts of Interest: The authors declare no competing interests.

Abbreviations

MPTP	1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine
MPP^+	1-Methyl-4-Phenylpyridinium Ion
MTT	3-(3,4-Dimehylthiazol-2-Yl)-2,5-Diphenyl-Tetrazolium Bromide
ER	Endoplasmic Reticulum
HUVEC	Human Umbilical Vein Endothelial Cells
L-DOPA	L-tyrosine to L-3,4-dihydroxyphenylalanine
MMP	Mitochondrial Membrane Potential
Mptp	Mitochondrial Permeability Transition Pore
PIP2	Phosphatidylinositol Biphosphate
PARP	Poly (ADP-Ribose) Polymerase
SNpc	Substantia Nigra Pars Compacta
TH	Tyrosine hydroxylase
TDAG8	T-Cell Death-Associated Gene 8
OGR1	The Ovarian Cancer G Protein-Coupled Receptor 1

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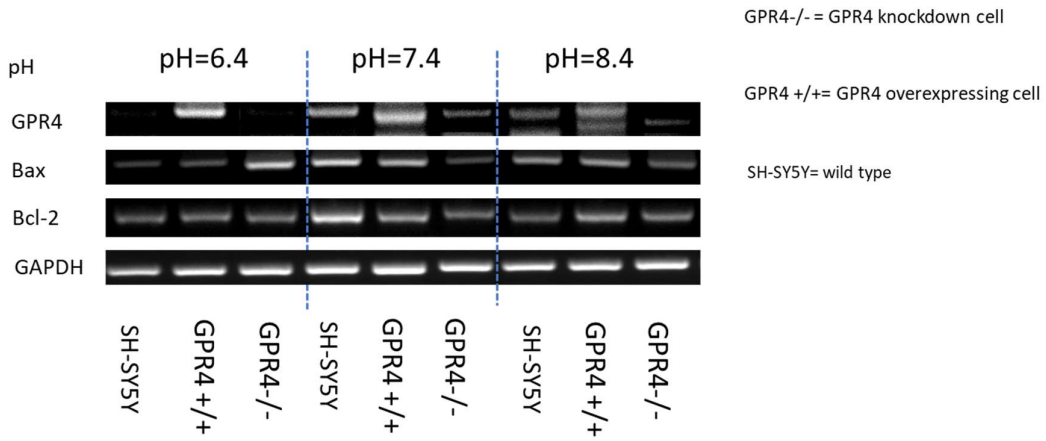
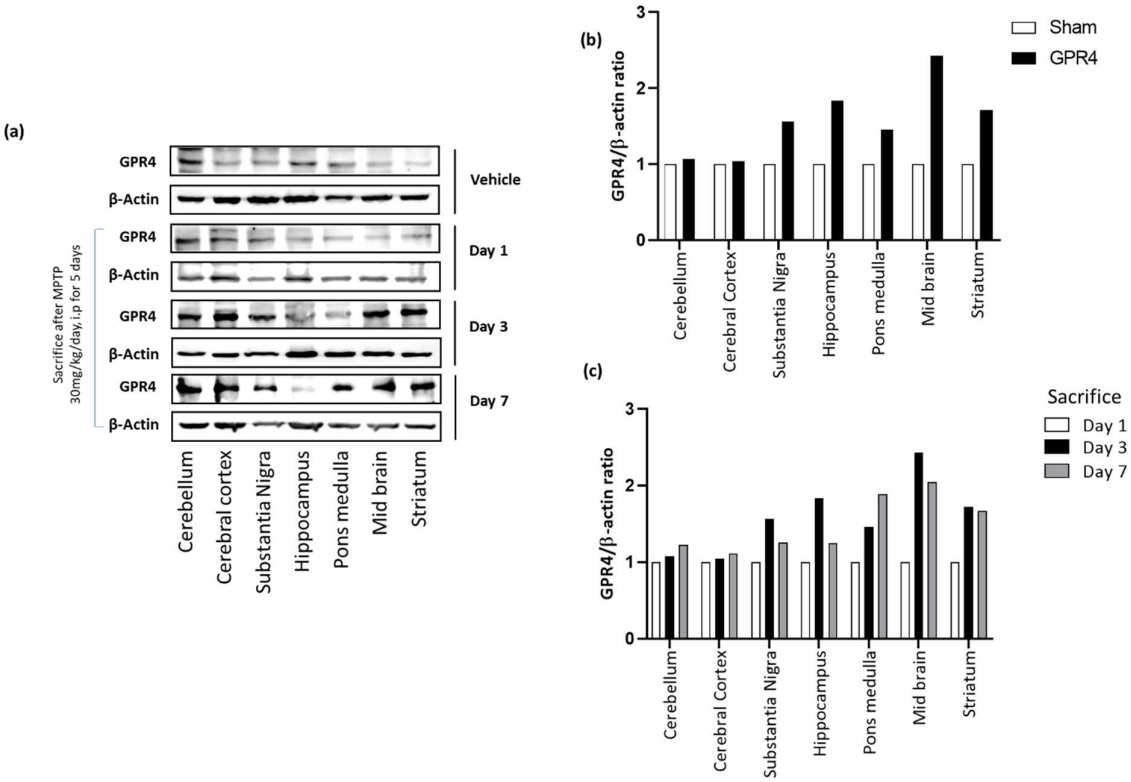


Supplementary data 1: pH condition changes the expression level of GPR4 and the expression of apoptotic markers. mRNA expression level of GPR4 and Bax, Bcl-2 apoptotic markers in the SH-SY5Y. SH-SY5Y stably expressing GPR4 and GPR4 knocked out stable SH-SY5Y cells treated with different pH mediums for 6 hours. GAPDH was used as internal control.



Supplementary figure 1: The effect of sub chronic administration MPTP, on the GPR4 Protein expression in different regions of mice brain. MPTP was treated (30mg/kg/day) for 5 days. Mice were sacrificed and tissue of different brain region was collected after 1, 3 and 7 days of last MPTP injection. (a). GPR4 protein expression in different regions of brain at different days after MPTP administration. (b). Densitometric analysis of the immunoblot data. β Actin ware used as an internal control.