Pharmacological Modulation of TRPM2 Channels via TRPM2 Pathway Leads to Neuroprotection in MPTP-induced Parkinson's Disease in Sprague Dawley Rats

Bhupesh Vaidya
National Institute of Pharmaceutical Education and Research
Harpinder Kaur
National Institute of Pharmaceutical Education and Research
Pavan Thapak
National Institute of Pharmaceutical Education and Research
Shyam Sunder Sharma
National Institute of Pharmaceutical Education and Research
Jitendra N Singh (jitnsingh@gmail.com)
National Institute of Pharmaceutical Education and Research https://orcid.org/0000-0003-0045-9295

Research Article

Keywords: Parkinson's Disease, MPTP, TRPM2 inhibitor, 2-APB, PJ-34, PARP

Posted Date: September 9th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-874639/v1

Abstract

Transient receptor potential melastatin-2 (TRPM2) channels are cation channels activated by oxidative stress and adenosine di-phosphate ribose (ADPR). Role of TRPM2 channels has been postulated in several neurological disorders, but, it has not been explored in animal models of Parkinson's disease (PD). Thus, the role of TRPM2 and its associated poly (ADP-ribose) polymerase (PARP) signalling pathways were investigated in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD rat model using TRPM2 inhibitor, 2-aminoethyl diphenyl borinate (2-APB) and PARP inhibitor, N-(6-Oxo-5,6-dihydrophenanthridin-2-yl)-(N,N-dimethylamino) acetamide hydrochloride (PJ-34). PD was induced by using a bilateral intranigral administration of MPTP in Sprague-Dawley rats, and different parameters were evaluated. An increase in the oxidative stress was observed, leading to the locomotor and cognitive deficits in the PD rats. PD rats also showed an increased TRPM2 expression in striatum and mid brain accompanied by reduced expression of tyrosine-hydroxylase (TH) in comparison to sham animals. Intraperitoneal administration of 2-aminoethyl diphenyl borinate (2-APB) and N-(6-Oxo-5,6-dihydrophenanthridin-2-yl)-(N,N-dimethylamino) acetamide hydrochloride (PJ-34) led to an improvement in the locomotor and cognitive deficits in comparison to MPTP-induced PD rats. These improvements were accompanied by a reduction in the levels of oxidative stress and an increase in TH levels in striatum and mid brain. In addition, these pharmacological interventions also led to a decrease in the expression of TRPM2 in PD in striatum and mid brain. Our results provide a rationale for the development of potent pharmacological agents targeting TRPM2-PARP pathway to provide therapeutic benefits for the treatment of neurological disease like PD.

Introduction

Transient Receptor Potential (TRP) ion channel family have a total of 28 different types of TRP channels classified into six subfamilies based on their primary amino acid structures: TRPA (ankyrin), TRPC (canonical), TRPM (melastatin), TRPML (mucolipin), TRPP (polycystin) and TRPV (vanilloid) [1]. Recently, TRP ion channels family has generated a lot of interest for its involvement in neurodegenerative diseases [2-4]. Transient Receptor Potential Melastatin 2 (TRPM2) channels, a sub-type of melastatin are Ca^{2+} permeable cation channels, which act as cellular redox sensor [5,6]. They are activated by reactive oxygen species (ROS), warm temperature and adenosine di-phosphate ribose (ADPR) [7,8].

Role of TRPM2 channels has been postulated in several neurological disorders such as Alzheimer's disease, bipolar disorders and cerebral ischemia [9-12]. The last two decades have led to the identification of mechanisms of the TRPM2 regulation and expression in the central nervous system [4,13]. TRPM2 channels are mainly expressed in the microglia, astrocytes and neuronal populations in the hippocampus, substantia nigra, striatum, and cortex [14-16]. TRPM2 channels are the integral membrane proteins, which have six transmembrane helixes. Hydrophobic residues between the S5 and S6 of these ion channels form the pore region for the entry of various monovalent and divalent cations such as Na^{+}, K^{+}, Ca^{2+} and Mg^{2+} [17]. It is a dual functional non-selective cation channel with enzymatic activity. It exhibits ADPR phosphatase activity because of the presence of enzymatic activity in its cytosolic C-terminal domain

((Nucleoside diphosphate linked moiety X)-type motif 9 (NUDT9)) which additionally serves as an agonist binding site for ADPR and the structurally analogous compounds [18]. TRPM2 channels activation leads to an increase in oxidative stress and contributes to neuronal cell death [19]. ADPR is the main gating molecule of TRPM2 which binds to its Nudix like domain with high specificity [20].

Poly (ADP-ribose) polymerase (PARP) enzymes in combination with Poly (ADP-ribose) glycohydrolase (PARG) generate ADPR through formation and hydrolysis of poly-ADPR when activated in response to DNA damage [21]. In neuronal cells, TRPM2 is activated by H_2O_2 through the formation of ADPR and increase the Ca^{2+}influx [5]. H_2O_2-induced increase of $[Ca^{2+}]_i$ influx is reduced or abolished by various PARP inhibitors, including PJ-34 [22,8]. This indicates that TRPM2 overexpression contributes towards oxidative stress-mediated neuronal death in Parkinson's disease (PD). A recent report has shown the increased expression of TRPM2 channels in the MPP^+ treated *in vitro* cells and substantia nigra pars compacta (SNpc) of the PD patients [19]. In PD due to death of the dopaminergic neurons, there is a generation of the oxidative stress and ADPR; which may activate TRPM2 channels [8]. However, there are no reports showing *in vivo* use of any pharmacological intervention to reduce TRPM2 mediated neuronal damage in PD. Therefore, in this study effects of pharmacological interventions, 2-APB, a TRPM2 inhibitor and PJ-34, a PARP inhibitor were investigated in the MPTP-induced PD model.

Materials And Methods

Animals

Male Sprague Dawley rats (280-300 g) were used for experimentation, as described elsewhere [23,24]. The animals were housed in a room maintained at approximately 24 ± 1°C temperature and humidity of 55 ± 5% with 12 h light/ dark cycle. Experiments were carried out in accordance with Committee for the Purpose of Control and Supervision on Experiments on Animals, Government of India; guidelines and approval of Institutional Animal Ethics Committee of National Institute of Pharmaceutical Education and Research, SAS Nagar, Punjab, India (IAEC/18/19 and IAEC/ 17/25).

Induction of PD and the experimental design

Rats were randomly assigned to the different groups such as Control (C), Sham (S), MPTP (M), MPTP + DMSO (M+V), MPTP + 2-APB (3 mg/kg) (M+A3), MPTP + 2-APB (10 mg/kg) (M+A10), Control + 2-APB (10 mg/kg) (A10), MPTP+ PJ-34 (3 mg/kg) (M+P3), MPTP+ PJ-34 (10 mg/kg) (M+P10) and Control + PJ-34 (10 mg/kg) (P10). A total of 6-10 animals per group were used for the experimentation. MPTP was administered to the rats by bilateral intranigral administration as described previously [24,23]. Animals were administered atropine sulphate (0.4 mg/kg, intraperitoneal) and sodium thiopental (50 mg/kg, intraperitoneal) before the surgical procedure [25]. Atropine sulfate was given before anaesthesia preceding surgical procedures in animals to reduce salivation, mucus production and other secretions in the airways. MPTP·HCl (100 µg in 1 µl of normal saline) was bilaterally infused at the coordinates of SNpc; anteroposterior (AP), - 5.0 mm from bregma; mediolateral (ML), ± 2.1 mm from midline;

dorsoventral (DV), - 7.6 mm from the dura mater with the help of stereotaxic apparatus (Stoelting, USA). Injection coordinates were verified using methylene blue dye. Rats in the sham-operated group were subjected to the same procedure with the infusion of 1 µl of normal saline instead of MPTP bilaterally into the SNpc [26].

Drug administration and treatment schedule

2-APB (Cat# D9754; Sigma Aldrich) and PJ-34 (Cat# P4365) were obtained from Sigma Aldrich, California, USA. Both 2-APB as well as PJ-34 reported “high” blood brain barrier permeability based on the binary artificial neural network ensemble (ANNE) classification model using GastroPlus® PBPK & PBBM Modeling and Simulation software. Though 2-APB is a broad spectrum TRP channel blocker, it has lower IC_{50} (0.82 µM) on TRPM2 channels and has also been used previously for other neurodegenerative conditions [27]. Therefore, doses were selected on the basis of previous reports [11,28,29]. The stock solutions of 2-APB (3 and 10 mg/kg) and PJ-34 (3 and 10 mg/kg) were freshly prepared in DMSO just before the injection. Intraperitoneal drug administration was done for two weeks, starting from day 7 (Fig. 1). Assessment of all the behavioral parameters was made between day 14 and 21; which was followed by the sacrifice of animals by decapitation on day 21. For the behavioural testing different tests were run on different days starting with Y-maze (day 15), open field test (day 16), rotarod test (day 17, 18) and passive avoidance (day 19, 20). After the behavioral study on day 21 animal were sacrificed; 50% animals were used for biochemical and western blotting study and 50% animals were used for immunohistochemistry study. All the other biochemical estimations and protein expression studies were performed after the sacrifice of these animals, as shown in Fig. 1.

Behavioral parameters assessment

Rotarod test

Motor function of the animal was evaluated using a rotarod test. For the training trial, animals were placed on a rotarod apparatus in separate lanes on rotating rod such that animals may walk forward to keep balance 8 rpm in 60 s. Animals were tested for four trials per day (20 min inter-trial interval) for the first day. Trial 3 was repeated if the animal fell off from the rod before the 60 s cut off time, but not more than four trials per animal were performed. Only those animals were included in the testing trial, which can stay on the rotating rod for the 60 s. A cut-off time of 300 s was taken for the animals during the test trial on the second day. The procedure was repeated for the total of three trials on the second day separated by 20 min inter-trial interval in case the animal falls before 300 s [30,31]. Fall latency was recorded for each animal and compared across the groups.

Locomotor activity

Locomotor activity of the animals was recorded for 10 min in an open field placed in a separate sound attenuated air-conditioned room with diffused light. Locomotor activity was assessed in terms of

distance travelled by the animals during this period. The distance travelled by each animal was recorded and normalized to the control [32].

Y-maze spontaneous alternation test

The Y-maze consisting of three arms, was used to access the short-term memory of the animals. Each rat was placed in one arm and allowed a free exploration for five-min trial in the Y-maze. The total number of arm entries and sequence of arm choices were recorded. An alternation was defined by the consecutive entry of the animal in the three arms without any repeated entries [33,11].

The percentage of relative alternations were calculated with the help of the formula

% alternation = [Number of alternations / (Number of total arm entries – 2)] × 100

Passive avoidance test

Passive avoidance apparatus (Columbus Instruments, USA) used for this test consisted of two compartments, an illuminated light compartment and a dark compartment separated by an automatically operated sliding door. Each rat was subjected to the initial habituation for a period of 15 s in the light compartment after which sliding door opened and it entered into the dark compartment. It was followed by an acquisition trial where the similar procedure was repeated beside it received a mild foot shock of 0.6 mA for 3 s through the grid floor in the dark compartment. The time taken by the rat to step into the dark compartment was recorded as initial trial latency. The rats which did not enter into the dark chamber within the cut-off time of 60 s were not considered for further experiments. After 24 h, retention trial was performed, and latency to step into the dark compartment was recorded as retention trial latency with 300 s as the cut-off time [34,32].

Malondialdehyde (MDA) estimation

After the behavioural experiments, the rats were sacrificed by decapitation. Striatum, midbrain, hippocampus and cortex were isolated and homogenized in PBS (pH 7.4). Homogenates were then used for the estimation of MDA levels to measure effect on MPTP on oxidative stress with the spreading of PD pathology as reported elsewhere [35].

Brain homogenate (0.1 ml) was mixed with 0.1 ml of 8.1% sodium dodecyl sulphate, 0.75 ml of 20% glacial acetic acid (pH=3.4), 0.75 ml of 0.8% thiobarbituric acid and 0.3 ml of distilled water. The mixture was vortexed and then heated on a water bath at 95° C for 1 h. MDA levels were estimated spectrophotometrically at a wavelength of 532 nm. Protein estimation was performed according to the Lowry method, and MDA content was expressed as μM of MDA per mg of protein [25,23,35].

Western blotting

Striatum and mid brain were isolated and homogenized in the lysis buffer containing 50 mM Tris-HCl, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 1 mM EDTA, 1 mM sodium

orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 µl/ml protease inhibitor cocktail, 5 mM of NaF, 10% SDS, 10% sodium deoxycholate and 1% of nonyl phenoxypolyethoxylethanol. Protein estimation was carried out according to the Lowry method. An equal amount of protein was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and then transferred on to a nitrocellulose membrane (Advanced Microdevices, India). After blocking with the 5% non-fatty dried milk for 1 h at room temperature, the membranes were incubated with a primary antibody against TH (Sigma-Aldrich Cat# T2928, RRID:AB477569, 1:6000) and TRPM2 (Sigma-Aldrich Cat# SAB2108271; 1:500) overnight at 4° C. The membranes were then washed with tris-buffered saline, 0.1 % tween-20 (TBST) and incubated with the horseradish peroxidase-conjugated secondary antibody (1:10000 dilution) for 60 min at room temperature. The membranes were then rinsed with TBST; the bound antibody was visualized by enhanced chemiluminescence method. ImageJ software was used to quantify the relative band intensities by densitometry and expression of the individual protein was normalized with β-actin [36,37].

Immunohistochemical analysis

Animals were anesthetized with thiopental sodium (50 mg/kg i.p.) and fixed by intracardial perfusion with 10% formaldehyde in PBS. The brains were rapidly removed and immersed in the same fixative overnight. The brain tissues were processed for paraffin embedding and then cut into 5-µm-thick coronal sections using the microtome (Leica Mikrosystem, Germany). These sections were obtained on the albumin coated slides, deparaffinized with xylene and rehydrated through graded concentrations of the alcohol. Immunohistochemistry was performed according to the manufacturer's instructions in the striatum and substantia nigra (ImmPRESS Excel Amplified Polymer Kit, Peroxidase, Cat# MP-7602). As it has been reported previously that progression of PD begins at dorsal striatum and later spreads to more ventral areas [38], TH immunohistochemistry of the whole striatum was performed. Briefly, antigen retrieval was performed using pH=6 citrate buffer followed by incubation in peroxidase blocking solution and horse serum albumin (2.5%). The sections were then incubated overnight at 4˚C in the primary antibody for TH (Sigma Aldrich, California, USA, Cat# T2928, 1:50). Then the primary antibody was removed, sections rinsed with TBS and further incubated with the secondary antibody at room temperature for 1 h. These sections were then again washed with TBS and incubated with polymer solution for 30 min. It was followed by incubation in the 3,3-o-diaminobenzidine solution at room temperature. The sections were then washed with TBS, counterstained with hematoxylin and striatum portion of the section was observed under a light microscope. Quantification was then performed using the Image J software. For quantification of striatal section, images were converted into 8 bit-grayscale followed by adjustment of threshold values to highlight the area in stained in brown because of DAB staining. Percent area was quantified using measure tool of Image J software and then normalized to average values of sham group. For the quantification of the IHC images from substantia nigra, total number of TH+ cells were counted using cell counter plugin of Image J software and values were normalized to average values of sham group [39].

Statistical analysis

Results were expressed as a Mean ± S.E.M. Statistical significance between the groups was evaluated using; one-way analysis of variance (ANOVA) followed by post hoc analysis using Tukey's test using the GraphPad Prism 7 software. $p<0.05$ was considered statistically significant.

Results

2-APB and PJ-34 attenuated the MPTP-induced alteration in cognitive and motor behavioural parameters

Parkinsonism in rats was induced by bilateral intranigral infusion of MPTP to trigger the selective degeneration of dopaminergic neurons in the substantia nigra. Rats in the sham-operated group were subjected to the same procedure with the infusion of normal saline instead of MPTP bilaterally into the SNpc. Intraperitoneal drug administration was done for two weeks, starting from day 7. Assessment of all the behavioral parameters was made between day 14 and 21; which was followed by the sacrifice of animals by decapitation on day 21. All the other biochemical estimations and protein expression studies were performed after the sacrifice of animals on day 21 (Fig. 1).

To assess effects on motor function, rats were evaluated in the rotarod and open field test. As expected, fall latency of MPTP-induced PD rats was significantly reduced as compared to the control and sham rats ($p<0.001$) (Fig. 2a). Vehicle treated MPTP-induced PD rats did not show any significant difference in fall latency as compared to the MPTP-induced PD rats. Treatment of 2-APB (3 and 10 mg/kg) significantly improved the fall latency as compared to the MPTP-induced PD rats ($p<0.001$). Moreover, PJ-34 (3 mg/kg) significantly reversed the MPTP-induced effect in rats ($p<0.001$). However, no improvement was observed at 10 mg/kg dose of PJ-34. The control animals which received treatment of 2-APB and PJ-34 did not produce any significant change in the fall latency in comparison to the control and sham rats [$F(9,65) = 24.31$, $p = 1.39 \times 10^{-17}$].

Distance travelled by the MPTP-induced PD rats was significantly reduced in comparison to the control and sham animals in the field ($p<0.001$) (Fig. 2b). Vehicle treated MPTP-induced PD rats did not show any significant difference in distance travelled in comparison to the MPTP-induced PD rats. Treatment of 2-APB (3 and 10 mg/kg) significantly increased the distance travelled in the MPTP-induced PD rats ($p<0.001$ and $p<0.05$) respectively. Moreover, PJ-34 treatment (3 and 10 mg/kg) to MPTP-induced PD rat also significantly increased the distance travelled in comparison to the MPTP-induced PD rats ($p<0.001$). The control animals which received treatment of 2-APB and PJ-34 didn't show any significant difference in comparison to the control and sham rats [$F(9,63) = 9.862$ $p = 2.97 \times 10^{-7}$].

In the test for the assessment of short term working memory using Y-maze spontaneous alternation test, MPTP-induced PD rats showed significantly reduced percent alternation in comparison to the control and sham rats ($p<0.001$) (Fig. 2c). Vehicle treated MPTP-induced PD rats did not show any significant difference in spontaneous alternation in comparison to the MPTP-induced PD rats. Treatment of 2-APB (3 and 10 mg/kg) significantly increased the percent alternation of the arm entries in the MPTP-induced PD rats ($p<0.001$). Moreover, PJ-34 (3 and 10 mg/kg) significantly increased the percent alternation in

comparison to the MPTP-induced PD rats ($p<0.001$). The control animals which received treatment of 2-APB and PJ-34 didn't show any change in the percent alternation in comparison to the control and sham rats [$F(9,80) = 26.32$, $p = 1.91 \times 10^{-20}$].

In the test for the assessment of fear conditioning memory using passive avoidance test, none of the groups showed any significant difference during the acquisition trial for the transfer latency into the dark compartment before receiving the electric shock [$F(9,78) = 0.9039$, $p = 0.5262$] (Fig. 2d).

In the retention trial, MPTP-induced PD rats showed a significant reduction in the transfer latency in comparison to the sham and control rats ($p<0.001$) (Fig. 2e). Vehicle treated MPTP-induced PD rats did not show any significant difference in the transfer latency in comparison to the MPTP-induced PD rat. Treatment with 2-APB (10 mg/kg) significantly increased the transfer latency in comparison to the MPTP-induced PD rats ($p<0.001$). However, PJ-34 (3 and 10 mg/kg) did not show any significant changes in the transfer latency in comparison to the MPTP-induced PD rats. The control animals which received treatment of 2-APB and PJ-34 didn't show any change in the transfer latency in comparison to the control and sham rats [$F(9,70) = 8.062$, $p = 4.33 \times 10^{-8}$].

These results indicate the ability of 2-APB and PJ-34 to positively modulate the MPTP-induced alterations in cognitive and motor performance of the rats.

Effect of 2-APB and PJ-34 on MDA levels

MPTP is known to increase oxidative stress levels contributing to neurodegeneration. Thus, to estimate the level of oxidative stress in different brain regions, we measured MDA levels in the striatum, mid brain, cortex and hippocampus. MPTP-induced PD rats showed a significant increase in the MDA levels in comparison to the control and sham rats in the striatum, midbrain, hippocampus and cortex ($p<0.001$) (Fig. 3a-d). Estimation of the oxidative stress in the different brains regions provided an estimate for the extent of MPTP toxicity after its intranigral administration. These findings correlate with the alteration in the behavioural parameters.

Vehicle treated MPTP-induced PD rats did not show any significant difference in MDA levels in comparison to the MPTP-induced PD rats. Treatment with 2-APB (3 mg/kg) also reduced the MDA levels significantly ($p<0.05$) in mid-brain, ($p<0.001$) in striatum, ($p<0.001$) in cortex and ($p<0.001$) in hippocampus region of the MPTP-induced PD rats. Also treatment with 2-APB (10 mg/kg) showed a significant reduction in the levels of MDA as compared to MPTP-induced PD animals in the striatum, midbrain, hippocampus and cortex ($p<0.001$ in striatum; $p<0.01$ in midbrain; $p<0.001$ in hippocampus; $p<0.001$ in cortex respectively).

Moreover, PJ-34 (10 mg/kg) also showed a significant reduction in the MDA levels as compared to the MPTP-induced PD rats ($p<0.001$). However, PJ-34 (3 mg/kg) didn't show a significant reduction in the MDA levels as compared to the MPTP-induced PD rats except in the striatum where a significant reduction was observed ($p<0.001$). The 2-APB and PJ-34 treatment to the control rats didn't show any

change in the MDA levels in comparison to the control and sham rats [Striatum: $F(9,34) = 44.23$, $p = 3.47 \times 10^{-16}$; Mid brain: $F(9,33) = 20.77$, $p = 3.67 \times 10^{-11}$; Hippocampus: $F(9,34) = 40.48$, $p = 1.35 \times 10^{-15}$; Cortex: $F(9,34) = 33.03$, $p = 2.92 \times 10^{-14}$].

These results suggest the ability of 2-APB and PJ-34 to reduce oxidative stress in different brain regions after MPTP administration.

Effect of 2-APB and PJ-34 on immunohistochemistry of TH in striatum and substantia nigra

We quantified the dopaminergic innervation of the striatum by measuring the TH immunoreactivity in striatal brain sections. MPTP-induced PD rats showed a significant reduction in the expression of TH in the striatum as compared to the sham rats ($p<0.001$) (Fig. 4 a,b). This reduced expression was significantly attenuated after the treatment of 2-APB and PJ-34 (10 mg/kg) in comparison to the MPTP-induced PD rats ($p<0.001$). The control animals which received treatment of 2-APB and PJ-34 didn't show any significant alteration in the TH levels in comparison to the sham rats [$F(7, 143) = 20.09$, $p = 1.35 \times 10^{-18}$]. These results show that treatment with 2-APB and PJ-34 attenuates the reduced TH expression in the striatum.

Similar results were obtained when TH positive neurons were evaluated in the substantia nigra. MPTP-induced PD rats showed a significant reduction in the TH positive neurons as compared to the sham rats in the substantia nigra ($p<0.001$) (Fig. 5 a, b). This reduced expression was significantly attenuated after the treatment of 2-APB (10 mg/kg) ($p<0.001$) and PJ-34 (10 mg/kg) ($p<0.05$). in comparison to the MPTP-induced PD rats The control animals which received treatment of 2-APB and PJ-34 didn't show any significant alteration in the TH positive neurons in comparison to the sham rats [$F(7, 61) = 14.50$, $p<0.0001$].

Effect of 2-APB and PJ-34 on protein expression of TH and TRPM2 in striatum and mid-brain

We evaluated the effects of 2-APB and PJ-34 on protein expression of TH (striatum) and TRPM2 (striatum and mid brain). TH expression was significantly reduced in the MPTP-induced PD rats in the striatum in comparison to the sham rats ($p<0.05$) as determined by western blot analysis (Fig. 6a, d). Treatment of 2-APB (10 mg/kg) and PJ-34 (10 mg/kg) significantly attenuated the reduced TH expression in the MPTP-induced PD rats ($p<0.05$), which also supports our immunohistochemistry observations. The control animals which received treatment of 2-APB and PJ-34 did not show any significant change in the TH expression in comparison to the sham rats [$F(7, 24) = 6.111$, $p = 0.0004$] (Fig. 6 a, d).

TRPM2 significantly increased in striatum in the MPTP-induced PD rats in comparison to the sham rats ($p<0.05$) (Fig. 6 B, E). 2-APB (10 mg/kg) significantly reduced the TRPM2 expression in the MPTP-induced PD rats ($p<0.05$). However, treatment of 2-APB (3 mg/kg) and PJ-34 (3 and 10 mg/kg) did not attenuate the TRPM2 expression in the comparison to MPTP-induced PD rats. The control animals which received treatment of 2-APB and PJ-34 did not show any significant alteration in the TRPM2 expression in comparison to the sham rats [$F(7, 24) = 3.125$, $p = 0.0172$].

TRPM2 significantly increased in mid brain in the MPTP-induced PD rats in comparison to the sham rats ($p<0.05$) (Fig. 6 c, f). 2-APB (3 and 10 mg/kg) significantly reduced the TRPM2 expression in the MPTP-induced PD rats ($p<0.05$). Moreover, treatment of PJ-34 (3 and 10 mg/kg) also significantly attenuated the TRPM2 expression in the comparison to MPTP-induced PD rats ($p<0.01$ and $p<0.05$ respectively). The control animals which received treatment of 2-APB and PJ-34 did not show any significant alteration in the TRPM2 expression in comparison to the sham rats [$F(7, 16) = 4.147$, $p = 0.0088$].

These results suggest that the pharmacological interventions under investigation are able to reduce the protein expression of TRPM2 in the MPTP-induced PD rats.

Discussion

The present study demonstrates the protective effect of 2-APB, a TRPM2 inhibitor and PJ-34, a PARP inhibitor in MPTP-induced PD model. TRPM2 is a prime candidate to contribute in this context, given its well-known permeability to Ca^{2+} and activation under conditions of elevated ROS. MPTP-induced PD model showed an alteration in the motor and cognitive functions along with an increase in the oxidative stress. Both 2-APB and PJ-34 were found to mitigate many of these behavioural consequences (locomotion and cognitive function) affected by MPTP treatment of rats. Increase in TRPM2 expression and biochemical changes were also attenuated by the TRPM2 inhibitors in MPTP-induced PD rats. Moreover, it has also been shown elsewhere that ADPR generated through PARP activates TRPM2 and promotes Ca^{2+} influx into the cells [40]. Therefore, the effect of PARP inhibition by PJ-34 on the TRPM2 mediated neuronal death in the MPTP-induced PD model was also investigated.

Intranigral MPTP injection has been shown in the earlier reports to induce PD by selective loss of dopaminergic neurons. It has been further reported that MPTP also reduces the TH expression and subsequently leads to motor and cognitive deficits in rats [41,25,23,24,42]. Our data is consistent with these reports of the MPTP administration, which have also shown a reduction in the open field and rotarod performance in the rodent model of PD. Use of pharmacological interventions 2-APB and PJ-34 targeting TRPM2 channel activity rescued the locomotor deficits seen in the MPTP-induced PD rats. However, no improvement was seen in MPTP-induced PD rats treated with higher doses of PJ-34 in the rotarod test possibly because of fatigue induction after administration of PARP inhibitors. It has been mentioned in the literature that the performance of the animals on the rotarod test could be altered by the fatigue even when the motor coordination is intact [43]. These results sum up the possible fatigue induction capability of PJ-34, which has also been reported elsewhere as well as when higher dose of PJ-34 was given to naive rats [44].

Besides motor symptoms, PD is often associated with non-motor symptoms such as memory loss and dementia because of the spreading disease pathology to the other brain regions, including cortex and hippocampus [45]. MPTP produced impairment in the fear conditioning and short term working memory as reported elsewhere [46,25]. Both 2-APB and PJ-34 showed a significant improvement in the cognitive parameters. However, only higher dose of 2-APB but not the PJ-34 could restore the deficits in the fear

conditioning memory. The possible explanation for this is that because PARP-1 is required for the consolidation of contextual fear memory, its inhibition may be detrimental for this kind of memory in the MPTP-induced PD rats. This observation is further supported by a report where micro-infusion of PJ-34 into the dorsal hippocampus has been associated with depletion of fear contextual memory [47].

Direct estimation of the ROS with a high degree of precision and accuracy is difficult in the tissue because of its short life span and reactivity with other cellular targets [48]. Therefore, we measured the ROS levels indirectly using MDA estimation, which is the by-product of lipid peroxidation. In line with the other studies, we also observed a significant increase in MDA levels in different regions of the brain after MPTP administration [25,49]. 2-APB and PJ-34 attenuated the MDA levels significantly indicating a reduction in the ROS levels, which is detrimental for the TRPM2 activation and associated neuronal death [50].

TH is a biomarker for PD because of its importance as a rate-limiting enzyme in the dopamine biosynthesis. Levels of the TH have been reported to decrease in the PD patients and as well as in the animal models of PD [51,52]. We confirmed these findings with the help of immunohistochemistry and western blotting for TH in the striatum. MPTP administration reduced the TH immunopositivity in the striatum which was restored by the higher dose of 2-APB and PJ-34. The neuroprotection accorded by these pharmacological agents as a result of reduced ROS levels and TRPM2 inhibition could be the reason for the partial increase in the TH levels and the associated nigrostriatal dopamine signalling in the MPTP-induced PD rats.

It has been shown that the TRPM2 is widely expressed in the human and as well as rat striatum [53,54]. From our findings, it is now established that both inhibitors exhibit the neuroprotective effects in the MPTP-induced PD animals. To delineate the mechanism of this neuroprotection, we looked into the effects of these pharmacological agents on the TRPM2 expression in the striatum as well as mid brain of the MPTP treated rats. 2-APB (10 mg/kg) treatment reduced the TRPM2 expression levels significantly in the striatum. A similar reduction though not statistically significant was observed after the PARP inhibition with PJ-34, which could be attributed to the reduced levels of ADPR, a TRPM2 activator. Similarly, in the mid brain increased expression of TRPM2 in MPTP-induced PD rats was significantly reduced both by 2-APB as well as PJ-34. It suggests that both direct and indirect inhibition of TRPM2 ion channels was effective in reducing its expression levels. However, the exact mechanism of this downregulation could not be established, and similar results have been reported elsewhere [11].

The rationale follows from the present work that has linked increased TRPM2 activation to pathology underlying neurodegenerative diseases. A common finding in neurodegenerative diseases is the occurrence of elevated oxidative stress and disruption of Ca^{2+} homeostasis as a consequence of influx via Ca^{2+} permeable membrane channels [2,55]. 2-APB is a broad spectrum calcium channel blocker has been shown to inhibit TRPM2, but also affects other members of TRP channels, including TRPM, TRPC and TRPV families [56]. Thus, even though 2-APB treatment mitigated MPTP behavioral outcomes, the extent to which these can be attributed to TRPM2 remains partially uncertain. Similarly, the role of PARP

inhibitor PJ-34 is complementary as TRPM2 is known to be activated by other mechanisms than those affected by PARP-mediated ADPR production. Furthermore, PARP-1 can also mediate its effects that are independent of TRPM2 (e.g. DNA repair and initiator of inflammatory responses) [57]. Additionally, it also sheds light on the possibility that each of these agents might also be acting via distinct and non-overlapping mechanisms. Moreover, it also points out that the blockade of other TRPC, TRPM and TRPV channels by 2-APB could also be highly relevant to PD pathology. However, 2-APB has an IC_{50} value of 0.82 μM on TRPM2 channels which is many folds in comparison to other targets such as TRPC1 (80 μM), TRPC5 (19 μM), TRPC6 (10.4 μM), TRPM7 (178 μM), TRPM8 (7.7 μM), IP3 receptor (42 μM), CRACM1 (8 μM) and CRACM3 (6 μM). It further points out at the possibility of TRPM2 blockade by 2-APB to be one of the major mechanism involved in PD pathology [27,58]. In the future, detailed mechanisms of neuroprotection accorded by TRPM2 blockade could be demonstrated with the help of knock out mice in context of PD.

In summary, these results provide novel insights into the possible pathogenic involvement of TRPM2 channels in the MPTP-induced PD. Furthermore, as it has been reported that TRPM2 channels play a pivotal role in other neurodegenerative disorders which are associated with oxidative stress, our findings could fill in the missing gaps in the pathophysiology of PD. This study provides the first evidence for the neuroprotective potential of the pharmacological interventions targeting TRPM2 channels in the *in vivo* model of PD (Fig. 7). Moreover, this study also provides a platform for future studies to explore the translational potential of the other pharmacological agents targeting TRPM2 in PD.

Abbreviations

TRPM2	Transient Receptor Potential melastatin-2
ADPR	Adenosine di-phosphate ribose
PD	Parkinson’s disease
PARP	Poly (ADP-ribose) polymerase
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
2-APB	2-aminoethyl diphenyl borinate
PJ-34	N-(6-Oxo-5,6-dihydrophenanthridin-2-yl)-(N,N-dimethylamino) acetamide hydrochloride
TH	Tyrosine-hydroxylase
TRP	Transient Receptor Potential
ROS	Reactive oxygen species
NUDT9	(Nucleoside diphosphate linked moiety X)-type motif 9
PARG	Poly (ADP-ribose) glycohydrolase
SNpc	Substantia nigra pars compacta
AP	Anteroposterior
ML	Mediolateral
DV	Dorsoventral
MDA	Malondialdehyde
ANOVA	One-way analysis of variance

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for Publication

Not applicable.

Compliance with Ethical Standards

Disclosure of potential conflicts of interest

The authors declare no competing interests.

Research involving Human Participants and/or Animals

Experiments were carried out in accordance with Committee for the Purpose of Control and Supervision on Experiments on Animals, Government of India; guidelines and approval of Institutional Animal Ethics Committee of National Institute of Pharmaceutical Education and Research, SAS Nagar, Punjab, India (IAEC/18/19 and IAEC/ 17/25).

Informed consent

Not applicable.

Data availability

All data supporting the conclusions of this manuscript are provided in the text, figures and tables.

Code availability

Not applicable

Author's contribution

BV: Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Writing - original draft; Writing - review & editing. **HK:** Investigation; Data curation; Formal analysis; Methodology; Visualization. **PT:** Western bloting Investigation and analysis; Visualization. **SSS:** Conceptualization; Resources; Supervision; Writing –final review & editing. **JNS:** Conceptualization; Data curation; Funding acquisition; Project administration; Resources; Supervision; Validation; Visualization; Roles/Writing - original draft; review & editing.

Funding

The present study was supported by financial support from start-up grant (R-12020/2017-HR) Department of Health Research, Ministry of Health and Family Welfare, Government of India. Also, authors received financial support from the National Institute of Pharmaceutical Education and Research, S.A.S. Nagar, Department of Pharmaceuticals, Ministry of Chemicals and Fertilizers, Govt. of India to carry out this work.

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Figures

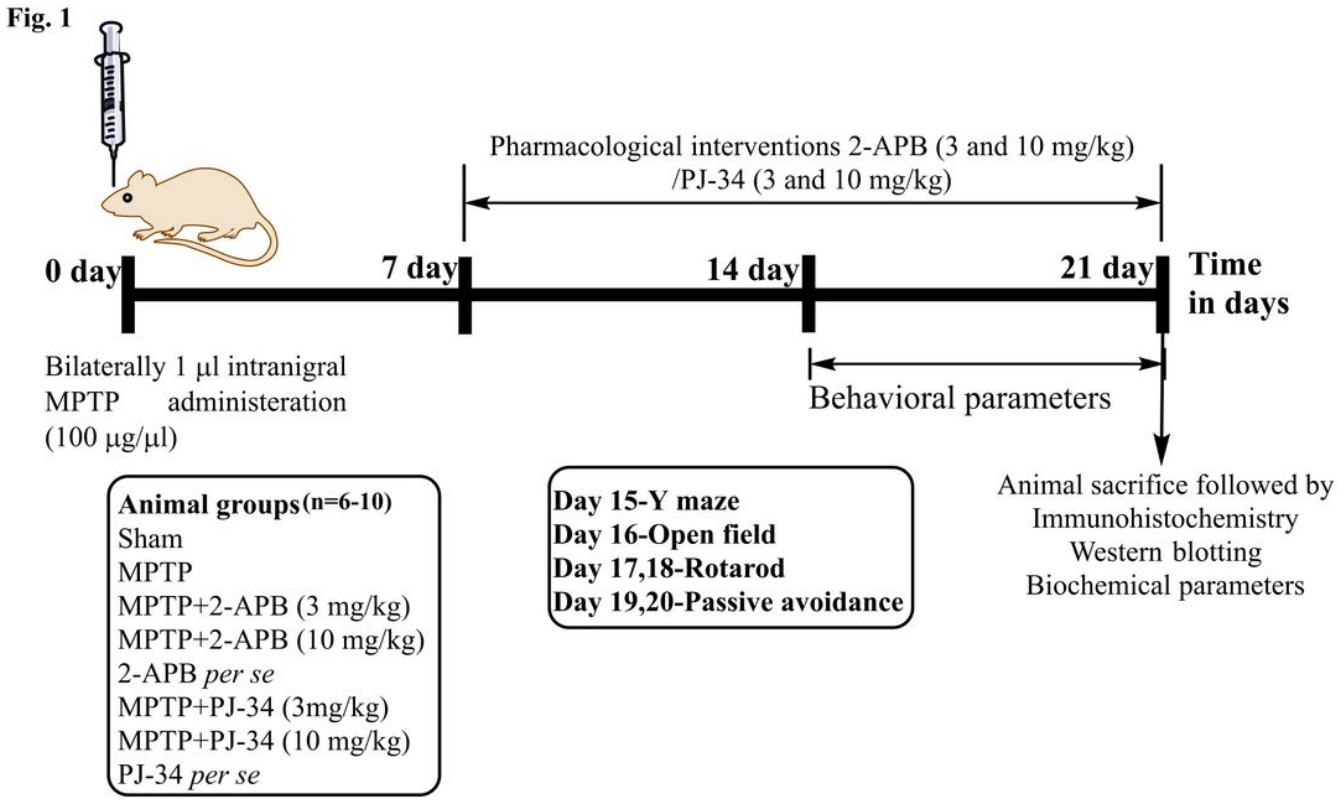


Figure 1

Schematic representation of the experimental design and treatment schedule has been shown. PD was induced in rats by bilateral intranigral administration of 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP; 100 µg/1µl). After 7 days of PD induction, 2-APB (3 and 10 mg/kg) / PJ-34 (3 and 10 mg/kg) were administered for 14 days to the MPTP-induced PD rats and control/sham rats. Then the Effect of 2-APB and PJ-34 on behavioral parameters, locomotor and cognitive deficits in MPTP-induced PD rat was investigated. After the behavioral study on day 21st animal were sacrificed; 50% animals were used for biochemical and western blotting and 50% animals were used for immunohistochemistry study.

Fig. 2

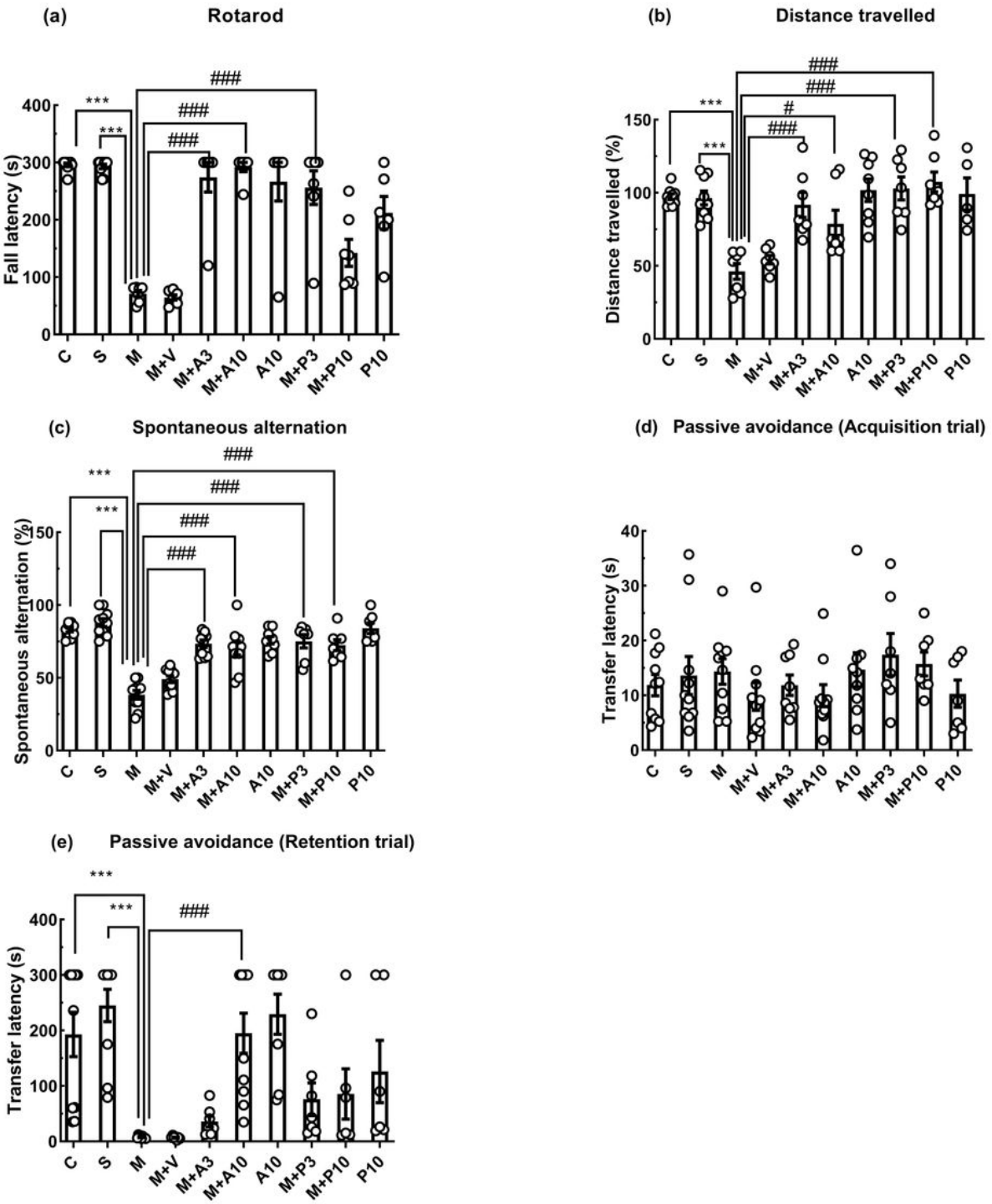


Figure 2

Effect of 2-APB and PJ-34 on behavioral parameters, locomotor and cognitive deficits in MPTP-induced PD rats (a) Fall latency during the rotarod test (n=10 for C, S; n=7 for M, M+V, M+A3, M+A10, A10, M+P3, M+P10 and n=6 in P10) (b) Normalized values of distance travelled in the open field test (n=10 for C, S; n=7 for M, M+V, M+A3, M+A10, M+P3, M+P10; n=8 for A10 and n=5 in P10) (c) Percent spontaneous alternation activity in the Y-maze test (n=10 for C S, M, M+V, M+A3, M+A10; n=9 for A10; n=7 for M+P3,

M+P10, P10) (d) Transfer latency in the passive avoidance test during the acquisition trial (e) Transfer latency in the passive avoidance test during the retention trial (n=10 for C, S, M+A10; n=9 for M; n=8 for A10; n=7 for M+V, M+A3, M+P3 and n=6 in M+P10, P10). Results were expressed as Mean ± SEM. ***p< 0.001 vs control and sham, #p<0.05, ###p<0.001 vs MPTP animals (One-way ANOVA).

Fig. 3

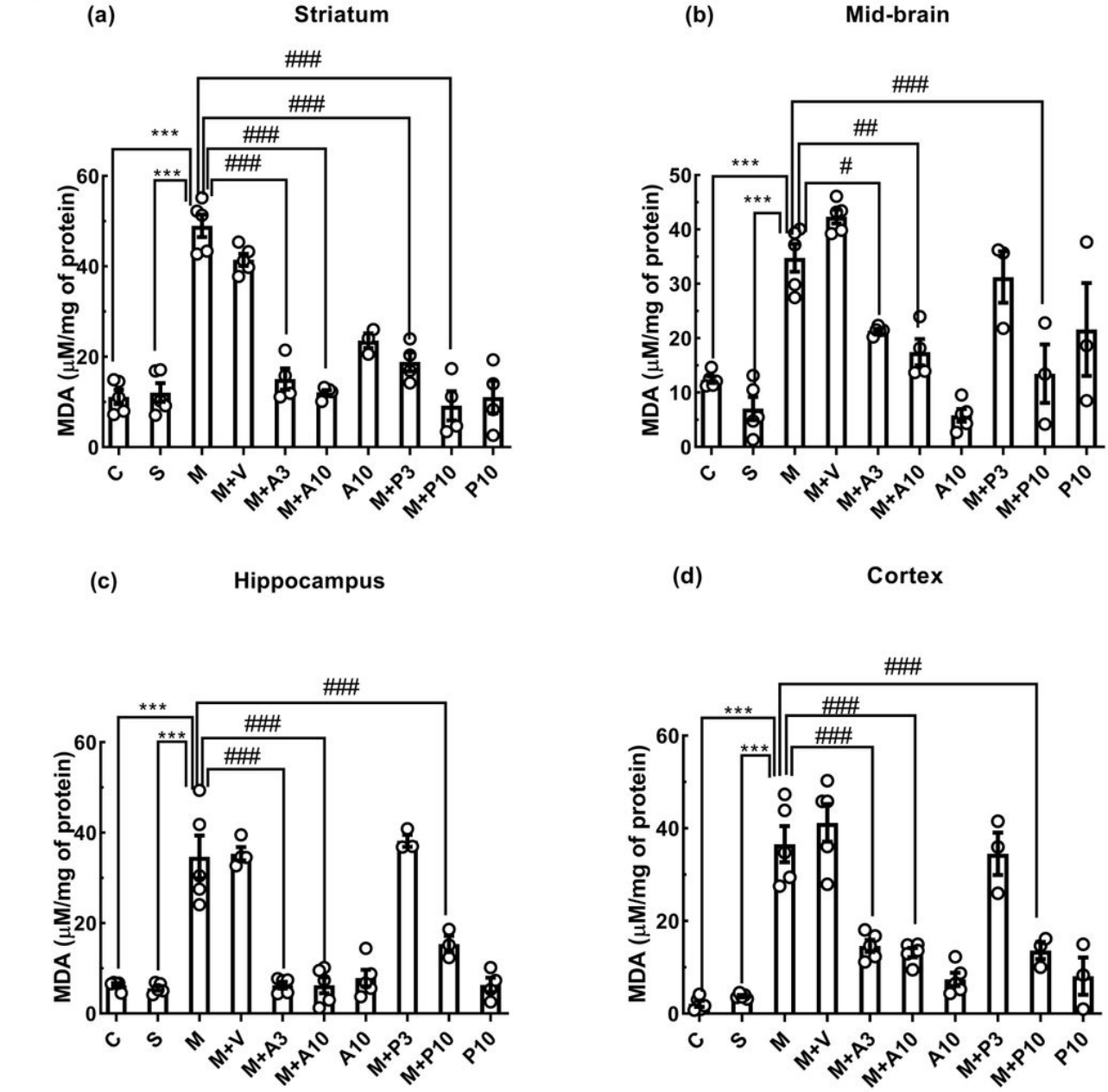


Figure 3

Effects of 2-APB and PJ-34 on MDA levels in the (a) striatum, (b) midbrain, (c) hippocampus, (d) cortex. Results are expressed as mean ± SEM. ***p< 0.001 vs control and sham, #p<0.05, ##p<0.01, ###p<0.001 vs MPTP animals (n=3-5; One-way ANOVA).

Fig. 4

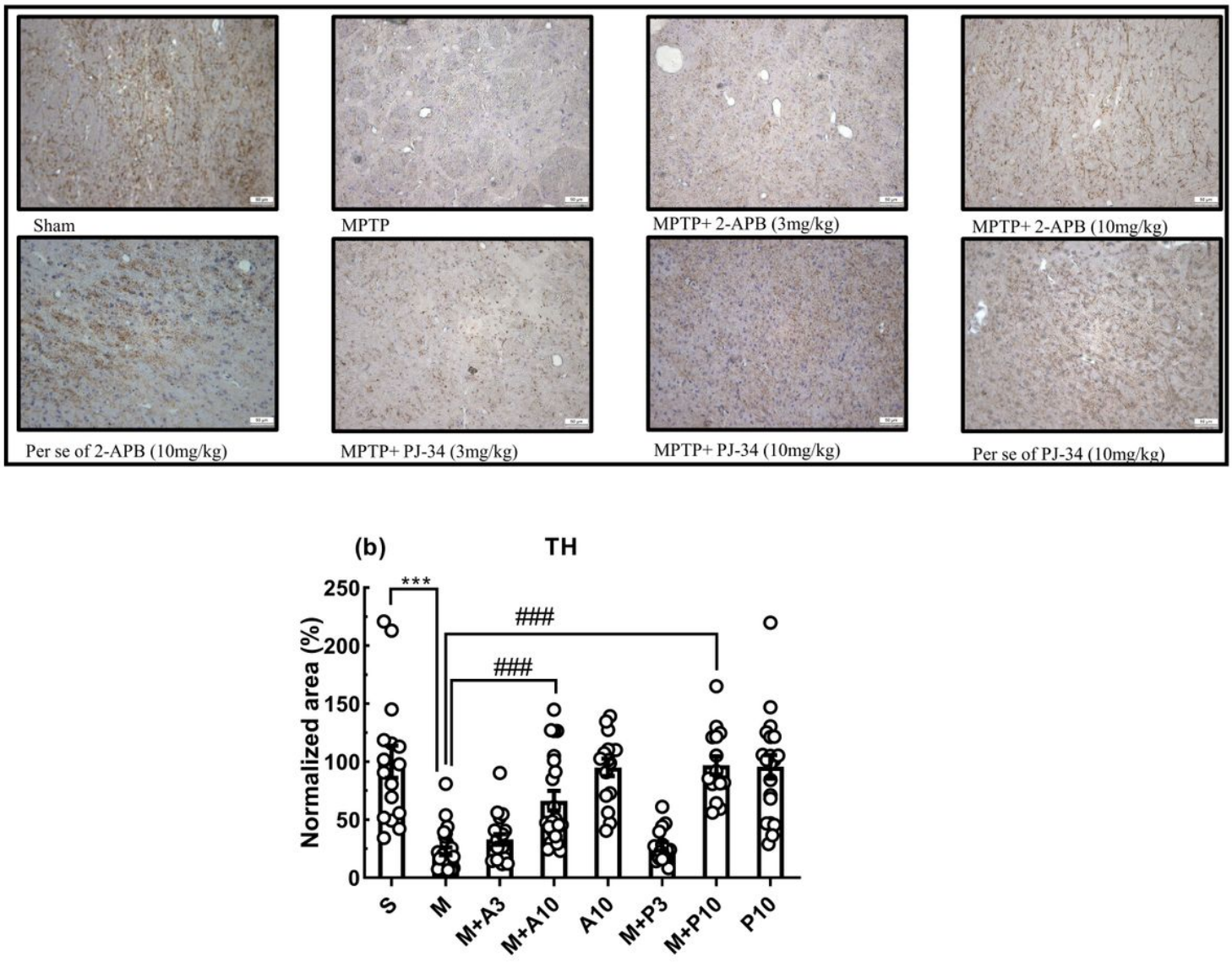
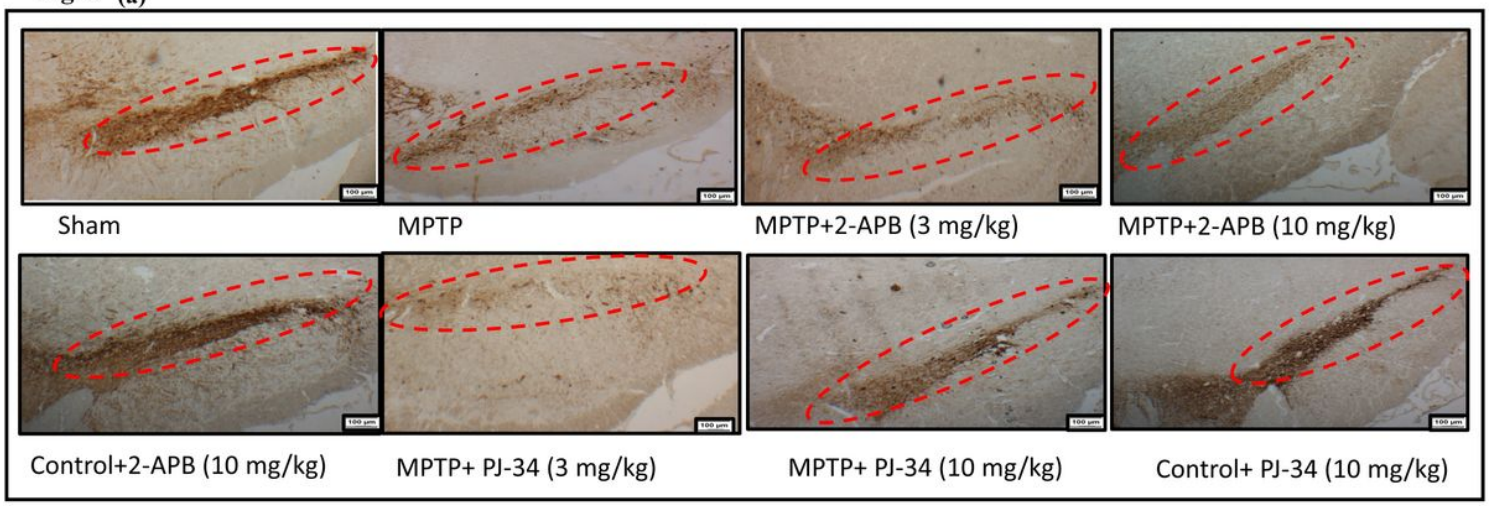


Figure 4

Protein expression of tyrosine hydroxylase (TH) (a) Immunostaining of TH in the striatum with 2-APB and PJ-34 in presence and absence of MPTP in rats. Images were taken at 20X magnification (n=3 animals per group, minimum of 6-7 images per animals) (scale bars = 50 μm). (b) Quantification of immunohistochemistry images for TH in striatum. Results were expressed as Mean ± SEM. ***p<0.001 vs sham animals; ###p<0.001 vs MPTP-induced PD animals (One-way ANOVA).

Fig. 5 (a)



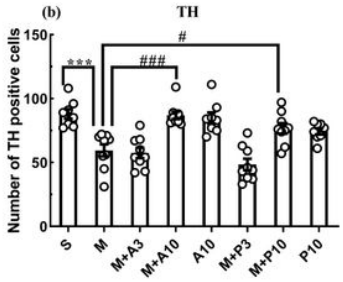


Figure 5

Protein expression of tyrosine hydroxylase (TH) (a) Immunostaining of TH in the substantia nigra with 2-APB and PJ-34 in presence and absence of MPTP in rats. Images were taken at 4X magnification (n=3 animals per group) (scale bars = 100 µm). (b) Quantification of immunohistochemistry images for TH in substantia nigra. Results were expressed as Mean ± SEM. (One-way ANOVA).

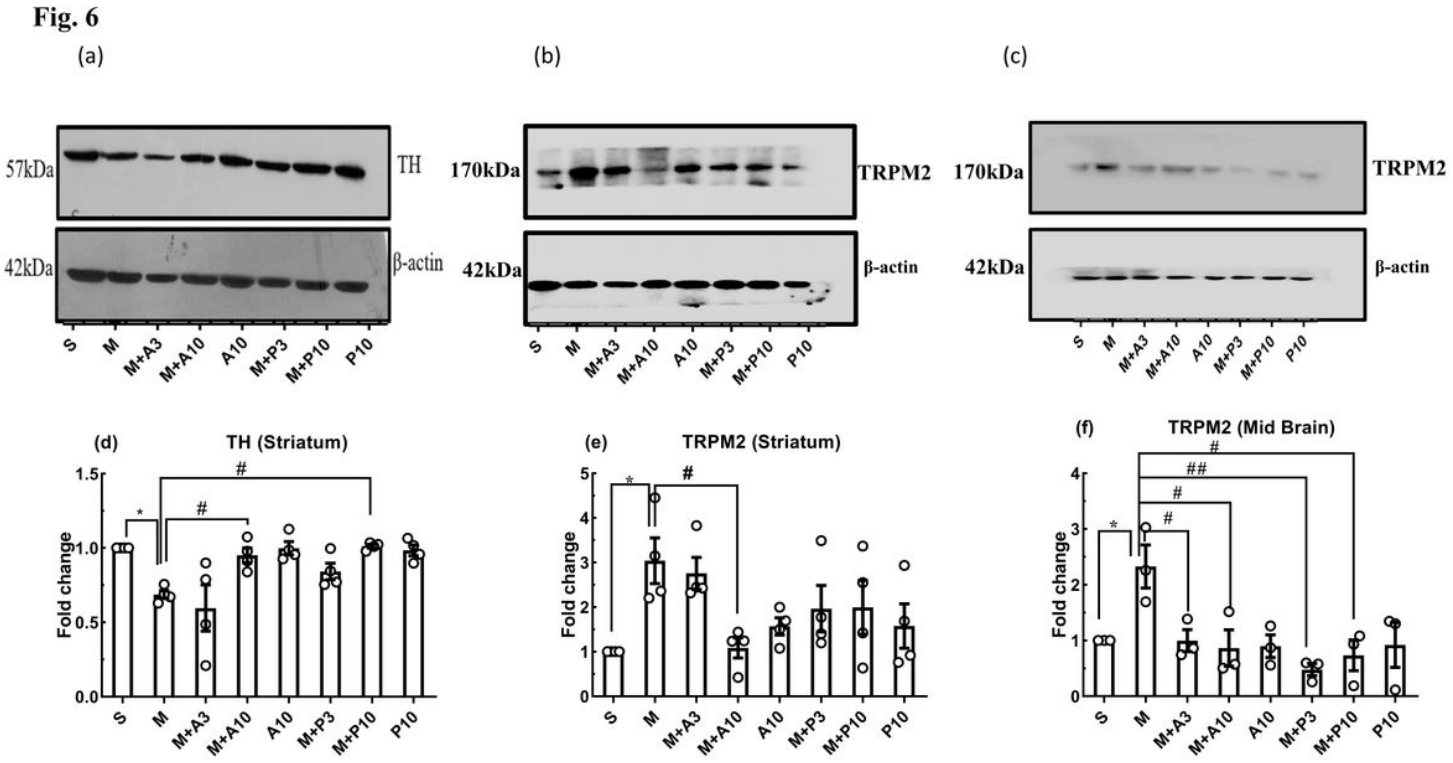


Figure 6

Western blot showing expression of (a) TH in the striatum, (b) TRPM2 in the striatum and (c) TRPM2 in the mid-brain. (n=3-4 per group). TH and TRPM2 blots were taken from different gels and ACTIN, TH and TRPM2 exposures with their antibodies are different. Quantification for blots is shown in (d) TH in striatum and (e) TRPM2 in striatum (f) TRPM2 in mid brain . Results were expressed as Mean ± SEM. *p<0.05 vs sham animals; #p<0.05, ##p<0.01 vs MPTP-induced PD animals (One-way ANOVA).

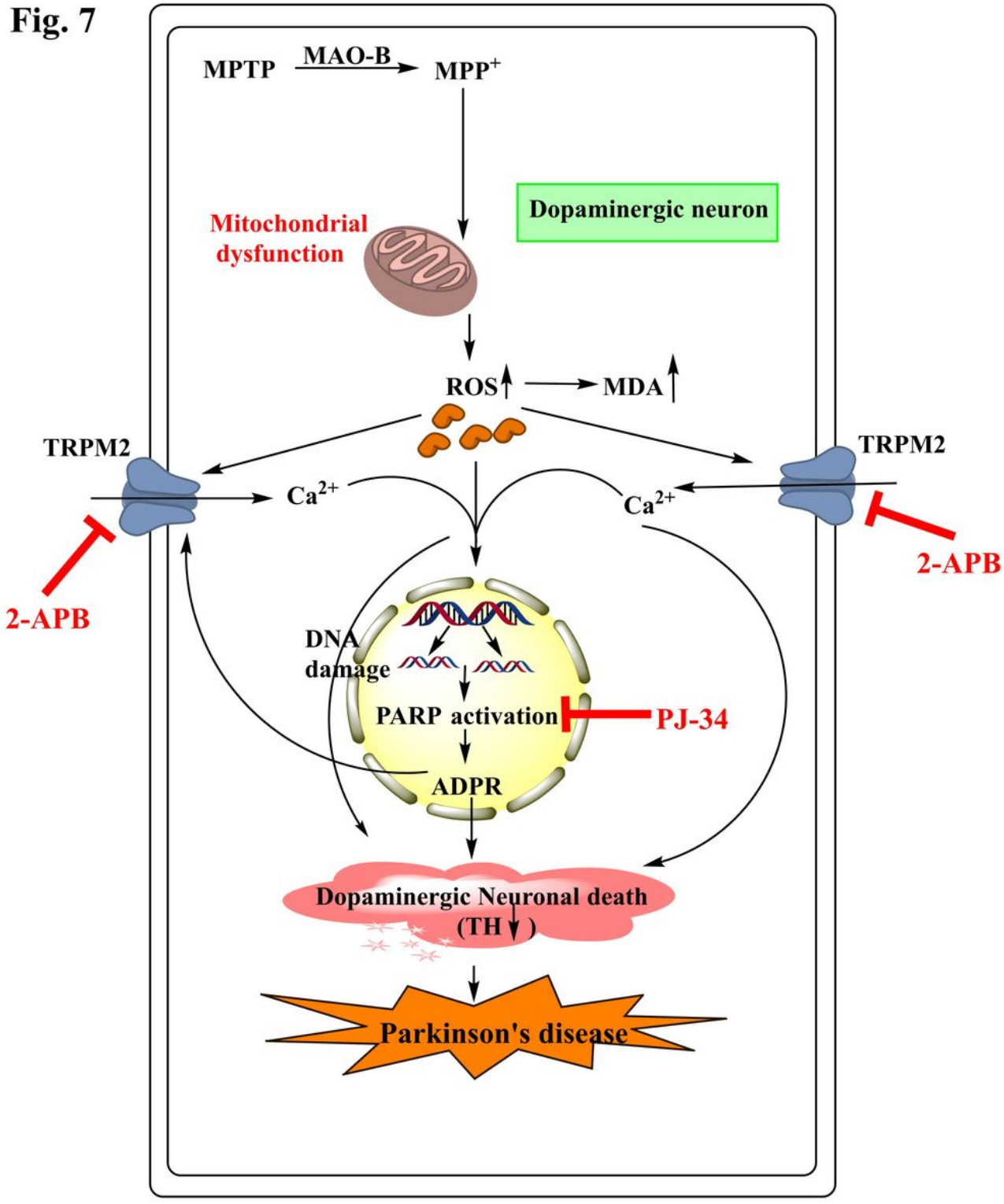


Figure 7

Schematic summary of the factors driving TRPM2 mediated neuronal death in PD. Induction of PD using MPTP leads to mitochondrial dysfunction, which generates reactive oxygen species (ROS) in the dopaminergic neurons. ROS overproduction activates the ROS-sensitive TRPM2 channels. ROS, along with Ca2+ influx via TRPM2 channels leads to DNA damage which causes PARP activation. The activation of the PARP enzyme produces ADPR, which further activates the TRPM2 channels and leads to

increased intracellular Ca2+ levels. These factors in totality cause the dopaminergic neuronal death further leading to motor and cognitive deficits. Administration of pharmacological agents targeting TRPM2 channels, TRPM2 inhibitor 2-APB and PARP inhibitor PJ-34 show neuroprotective effect by reducing MPTP-induced toxicity and increased oxidative stress. These agents lead to an improvement in the locomotor and cognitive function and restore dopaminergic function by the restoration of the tyrosine hydroxylase levels. 2-APB additionally works by reducing the TRPM2 expression in the MPTP model of PD.