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TRPM2 promotes neurotoxin MPP+/MPTP-induced cell death

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Abstract

In neurons, Ca^{2+} is essential for a variety of physiological processes that regulate gene transcription to neuronal growth and their survival. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 1-methyl-4-phenylpyridinium ions (MPP^+), are potent neurotoxins that selectively destroys the dopaminergic (DA) neurons and mimics Parkinson's disease (PD) like symptoms, but the mechanism as how MPP^+/MPTP effects DA neuron survival is not well understood. In the present study we found that MPP^+ treatment increased the level of reactive oxygen species (ROS), that activates and upregulates the expression and function of melastatin-like transient receptor potential (TRPM) subfamily member, TRPM2. Correspondingly, TRPM2 expression was also increased in substantia nigra of MPTP-induced PD mouse model and PD patients. ROS-mediated activation of TRPM2 resulted in increased intracellular Ca^{2+}, which in turn promoted cell death in SH-SY5Y cells. Intracellular Ca^{2+} overload caused by MPP^+-induced ROS also affected calpain activity, followed by increased caspase 3 activities and activation of downstream apoptotic pathway. On the other hand, quenching of H_2O_2 by antioxidants, resveratrol (RSV), or N-acetylcysteine (NAC) effectively blocked TRPM2 mediated Ca^{2+} influx, decreased intracellular Ca^{2+} overload, and increased cell survival. Importantly, pharmacological inhibition of TRPM2 or knockdown of TRPM2 using siRNA, but not control siRNA, showed increased protection by preventing MPP^+-induced Ca^{2+} increase and inhibited apoptosis. Taken together, we show here a novel role for TRPM2 expression and function in MPP^+-induced dopaminergic neuronal cell death.

Keywords

TRPM2; oxidative stress; ROS; calcium; MPTP/MPP^+; apoptosis

Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder and loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) underlies the main motor

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symptoms of Parkinson's disease [1]. DA neuronal protection or prevention of DA neuron degeneration has been identified as possible therapeutic mechanism to prevent/treat PD. Although the detailed mechanisms responsible for dopaminergic neuronal death in PD is not well established, one crucial factor is shown to be oxidative stress. Oxidative stress is known to be a major contributing factor that leads to disturbed mitochondrial membrane potential and the cascade leading to degeneration of dopaminergic neurons in PD [2,3]. Importantly, PD patients, exhibits increased reactive oxygen species (ROS) and lipid peroxidation, reduced mitochondrial complex 1 activity in the SNpc region, all of which are related to oxidative stress.

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a potent neurotoxin has been shown to selectively destroy the DA neurons in humans, sub-human primates, and lower animals that mimics PD like symptoms [4]. MPTP is rapidly converted into 1-methyl-4-phenylpyridinium ions (MPP^+) when MPTP crosses the blood brain barrier into the brain [5]. Moreover, MPP^+ is then selectively taken up by dopaminergic neurons via the high-affinity dopamine transporter (DAT) and is concentrated within the mitochondria where it acts to inhibit electron transport chain, decrease mitochondrial membrane potential, and induce disturbances in Ca^{2+} homeostasis, which could eventually lead to neuronal loss [6,7]. Importantly, neurotoxins including MPP^+ have been shown to activate ROS-dependent cascade during dopaminergic cell death [6,8]. Recent studies have also demonstrated that under oxidative stress, high levels of ROS, including free radicals such as superoxide (O_2^-), hydroxyl radical (HO^-) and hydrogen peroxide (H_2O_2) are produced, which may modify certain proteins, lipids and DNA structures that consequently cause severe cell damages and eventually cell death [9]. Interestingly, evidence also shows that ROS-induced endothelial dysfunction is often preceded by an alteration of endothelial $[Ca^{2+}]_i$ [9,10], which could serve as an important second messenger to trigger apoptosis and cell death. Previously we have reported that transient potential canonical channel 1 (TRPC1) is perilous for neuronal survival and that treatments with the neurotoxin 1-methyl-4-phenylpyridinium (MPP^+) attenuates the TRPC1 expression in SH-SY5Y and PC12 cells [6]. TRPC1 is important for SOCE and thus could be important for modulating ER calcium levels that is also essential for the survival of dopaminergic neurons [11,12]. However, calcium channels that are activated by oxidative stress could also play a vital role in neuronal survival.

Melastatin-like transient receptor potential channel 2 (TRPM2) channels have gained much attention in recent years, which are Ca^{2+} permeable nonselective channels that are highly expressed in the brain [13,14] and have been implicated to oxidative stress. TRPM2 is potentiated by oxidative stress, ADPR/NAD^+ metabolism TNF-α and heat [15] which results in increased intracellular Ca^{2+} ($[Ca^{2+}]_i$) concentrations in various cell types [16–19]. In addition H_2O_2 has been also shown to directly stimulate TRPM2 channel [20,21]. Importantly, in rat cortical neurons, Ca^{2+} influx through TRPM2 causes a positive feedback loop of ROS production, which leads to the loss of these neuronal cells [22]. However, the role of TRPM2 underling neuronal degeneration of dopaminergic neurons, especially upon neurotoxin MPP+ treatment as well as its mechanism, has not been identified. In light of the above observations, we hypothesized that neurotoxins-induced oxidative stress will activate TRPM2 leading to Ca^{2+} entry that increase in calpain activation that induces apoptosis. The data presented here demonstrates that MPP^+ induces accumulation of H_2O_2, which in turn

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activates the TRPM2 mediated Ca^{2+} influx necessary for the initiation of apoptosis. Furthermore, TRPM2 knockdown prevented MPP^{+}-induced cellular death by inhibiting apoptosis in SH-SY5Y neuronal cells, whereas overexpression increased cell loss. Hence, inhibition of TRPM2 could be an effective mean to protect cells against degeneration induced by dopaminergic neurotoxin MPP^{+}.

Material and methods

Cell culture, transfections, viability and hydrogen peroxide levels assays

SH-SY5Y cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured/maintained at 37°C with 95% humidified air and 5% CO_2. SH-SY5Y cells were differentiated by the addition of retinoic acid for 6 days and used for the experiments [6]. For transient transfection of SH-SY5Y cells were transfected with TRPM2siRNA (Ambion, NY) or scrambled control siRNA (Ambion negative control siRNA #1) Transfection use Lipofectamine RNAiMAX Reagent(Life technologies, NY) in Opti-MEM medium as per supplier's instructions. For viability assays cells were seeded on 96-well plates at a density of 0.5×10^{5} cells/well. The cultures were grown for 24 hours under different conditions and cell viability was measured by using the MTT method as described in [23]. The Fluorescent Hydrogen Peroxide Assay Kit (Sigma, MO) was used for hydrogen peroxide levels analysis as per supplier's instructions.

Animals and human brain samples

8–10 months old male C57BL/6 mice (Charles River Laboratories, USA) were used for these experiments. All animals were housed in a temperature controlled room under a 12/12-hour light/dark cycle with ad libitum access to food and water and experiments were carried out as per the institutional guidelines for the use and care of animals. For PD mice model, eight to ten months older mice were challenged with either MPTP (MPTP-HCl, 25 mg/kg per injection, i.p.) or saline for 5 consecutive days at 24-hour intervals. Mice were sacrificed 7 days after the last MPTP injection and substantia nigra was isolated as described in previous studies [6]. Mice were sacrificed 7 days after the last MPTP injection and substantia nigra was isolated. Frozen and Paraffin-embedded blocks of postmortem human substantia nigra samples of control (6 samples, males with age ranging from 65–72) and Parkinson's disease patients (8 samples, males/females ages 60–72, with severe motor neuron deficit) were obtained from UK Parkinson's foundation as well as Udall Parkinson Center, Philadelphia. All human subjects were approved by the institutional IRB.

Electrophysiology

For single cells recording, coverslips with SH-SY5Y cells were transferred to the recording chamber and perfused with an external Ringer's solution of the following composition (mM): NaCl, 140; KCL, 5; $MgCl_2$, 1; $CaCl_2$, 1; Hepes, 10; Glucose, 10; pH 7.4 (NaOH). The patch pipette had resistances between 3 –5 m after filling with the standard intracellular solution that contained the following (mM): cesium methanesulfonate, 145; NaCl, 8; $MgCl_2$, 5; Hepes, 10; EGTA, 1; pH 7.3 (CsOH). The Ca^{2+} concentration was adjusted to 1uM. Cells were held at a potential of −60 mV, and current–voltage (I–V) relations were obtained from voltage ramps from −80 to +80 mV applied over 200 ms. For brain slice recording, 2–4

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weeks old mice were sacrificed. Brain sections (400 μm) were cut using a vibrating blade microtome (VT1000S; Leica). Recording electrodes were filled with (in mM) 100 CsCL, 0.6 EGTA, 2 $MgCl_2$, 8 NaCl, 33 HEPES, 2 $ATPNa_2$, 0.4 GTPNa and 7 phosphocreatine, pH 7.4. The extracellular solution comprised (in mM) 130 NaCl, 24 $NaHCO_3$, 3.5 KCl, 1.25 NaH_2PO_4, 2.5 $CaCl_2$, 1.5 $MgCl_2$ and 10 glucose, saturated with 95% O_2 and 5% CO_2, pH 7.4. TTX was added in extracellular solution. All electrophysiological experiments were filtered at 2 kHz, digitized at 10 kHz, acquired and analyzed using pCLAMP 10 software (Molecular Devices).

Calcium Measurements

Cells were incubated with 2 μM fura-2 (Molecular Probes) for 45 min, washed twice with Ca^{2+} free SES (Standard External Solution, include: 10 mM HEPES, 120 mM NaCl, 5.4 mM KCl, 1 mM MgCl2, 10 mM glucose, pH 7.4) buffer. For fluorescence measurements, the fluorescence intensity of Fura-2-loaded control cells was monitored with a CCD camera-based imaging system (Compix) mounted on an Olympus XL70 inverted microscope equipped with an Olympus 40× (1.3 NA) objective. A monochrometer dual wavelength enabled alternative excitation at 340 and 380 nm, whereas the emission fluorescence was monitored at 510 nm with an Okra Imaging camera (Hamamatsu, Japan). The images of multiple cells collected at each excitation wavelength were processed using the C imaging, PCI software (Compix Inc., Cranbery, PA), to provide ratios of Fura-2 fluorescence from excitation at 340 nm to that from excitation at 380 nm (F340/F380) [24,25]. Fluorescence traces shown represent [Ca2+]i values that are averages from at least 30–40 cells and are a representative of results obtained in at least 3–4 individual experiments.

Assessment of apoptosis

The vibrant apoptosis assay kit was used to assess apoptosis as per the manufacturer's instructions (Molecular Probes, Eugene, OR). This kit detects dead cells with Annexin V and propidium iodide (PI) staining. The cells were visualized by flow cytometry (using a BD LSR II; Becton Dickinson, San Jose, CA). The total number of apoptotic cells was counted, and the percentages of cells exhibiting apoptosis were calculated [26].

Western blot analysis

Rabbit polyclonal Ab anti-TRPM2 (Cat #: ab101738) was purchased from Abcam (Cambridge, MA). Goat polyclonal Abs against β-actin (Cat #: sc-1616) was obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Rabbit monoclonal Ab against TH (Cat #: 2792), cleaved caspase3 (Cat #: 9664), Bak (Cat #: 2772) and Bid (Cat #: 2002) were purchased from Cell Signaling Technology (Cambridge, MA). Cells were stimulated as indicated in the figure followed by the addition of RIPA buffer to obtain cell lysates from both SH-SY5Y cells, and SN region of the brain. Protein concentrations were determined, using the Bradford reagent (Bio-Rad), and 25 ug of lysates were resolved on NuPAGE 4–12% Bis-Tris gels (Invitrogen, Carlsbad, CA) followed by Western blotting.

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Calpain activation and Caspase 3 activity

Two million cells were grown on 35-mm plates and were treated with 500 μM MPP^+ or 24 hours. The cells were lysed and protein concentration was measured. The cell lysate was diluted using extraction buffer and the calpain activation was measured according to the manufacturer's instructions (Abcam, MA). The samples were analyzed at an excitation of 400 nm and emission at 505 nm using Multiskan spectrum fluorimeter (Thermo lab systems) and the colorimetric reading was normalized with the respective total protein concentrations. Caspase 3 activity was measured using abcam Caspase 3 assay kit. One million cells were isolated using cell lysis buffer and the liquid fraction was used to analysis the caspase 3 activity as manufacturer's instructions. The sample absorbance was measured at 450nm and graph was plotted using absorbance value [27].

Confocal Microscopy

For immunofluorescence assays, cells were grown overnight on coverslips and fixed for 30 minutes using 4% paraformaldehyde, permeabilized using methanol and blocked with 5% horse serum for 1 hr. For TRPM2 staining, cells were treated with TRPM2 antibody 1:100 dilution and washed and labeled with Alex 488 linked anti-rabbit secondary antibody 1:100 dilution for 1 hr. Confocal images were collected using a MRC1024-krypton/argon laser scanning confocal equipped with a Zeiss LSM 510 Meta photomicroscope.

Statistical analysis

Data analysis was performed using Origin 9.0 (Origin Lab, Northampton, MA) and prism Graph pad 5.0. Students paired or unpaired *t* test or analysis of variance (ANOVA) was used for statistical analysis as appropriate; *N* number in the text represents the cells. Differences in the mean values were considered to be significant at $p < 0.05$.

Results

MPP^+ induces hydrogen peroxide accumulation, intracellular Ca^{2+} overload followed by neuronal death

Oxidative stress is known to be a major contributing factor leading to the degeneration of dopaminergic neurons in PD [28]. We first studied the effect of MPP^+ on cell death of dopaminergic cells. MPP^+ treatment showed a time and concentration dependent increase in cell death in SH-SY5Y cells (Fig. 1A), which is consistent with previous reports [29,6]. To define the role of ROS in MPP^+-induced apoptosis, we further studied the effect of MPP^+ on accumulation of H_2O_2 in SH-SY5Y cells (Fig. 1B). Since MPP^+ treatment induced a time dependent H_2O_2 accumulation (as observed by the release of H_2O_2) in SH-SY5Y cells, it suggest that MPP^+ induced intracellular H_2O_2 generation especially after MPP^+-treatment (Fig 1B). At the same time, exogenous treatment with H_2O_2 attenuated cell survival significantly in a dose dependent manner (Fig. 1C). Ca^{2+} plays a significant role in H_2O_2-induced cell death [30], thus we focused our attention to Ca^{2+} entry. As shown in Fig. 1D, E, Ca^{2+} influx was increased after H_2O_2 application in SH-SY5Y cells. Most importantly, Ca^{2+} influx facilitated by MPP^+ was also time dependent. Previous study from our lab had shown that MPP^+-induces apoptosis [31], hence we studied the amount of apoptotic cells by

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percentage using PI/annexin V staining analyzed on a flow cytometer (Fig 1F, G). Consistent with our previous study, the percentage of apoptotic cells was significantly higher in MPP^+ treated cells as compared to control and similar results were obtained in the presence of H_2O_2 (data not shown). Moreover, caspase 3 activity was consistently increased in cells with MPP^+ treatment (Fig 1H). Collectively, these findings implicate that MPP^+ -induces H_2O_2 accumulation which in turn activates Ca^{2+} influx leading to intracellular Ca^{2+} overload needed for increased caspase activation and/or apoptosis.

Hydrogen peroxide induces Ca^{2+} influx via the TRPM2 channel

To further characterize the source of Ca^{2+} influx, whole cell recordings were performed. Importantly, addition of H_2O_2 in SH-SY5Y cells showed a non-selective inward current that reversed between 0 and −5mV (Fig. 2A, B). The properties of the inward current were similar as that observed with TRPM2 [16,21], suggesting that H_2O_2 activates TRPM2 currents. To establish that indeed the currents are mediated via TRPM2, electrophysiology experiments using flufenamic acid (FFA) were performed. Flufenamic acid and *N*-(*p*-amylcinnamoyl) anthranilic acid (ACA) are known TRPM2 blockers [32] [33] which can significantly inhibit the Ca^{2+} influx induced by TRPM2 channels. Moreover, addition of FFA significantly attenuated H_2O_2 induced Ca^{2+} influx (Fig 2A–C). Again, H_2O_2 induced similar non-selectively inward currents with channel properties that were similar to those previously observed with TRPM2 channels and whose currents were significantly inhibited by FFA or ACA (Fig. 2A–C) [18,16]. In addition, Ca^{2+} influx induced by H_2O_2, was also significantly blocked by the addition of FFA or ACA (Fig. 2D, E). To further establish the identity of the H_2O_2-induced Ca^{2+} influx ADP-ribose was used, which has been shown to directly activate TRPM2 channels [34,35]. Similar to the addition of H_2O_2, addition of ADP-ribose also showed a non-selective inward current (Fig. 2F, G), suggesting TRPM2 channels exist in SH-SY5Y cells.

To establish that TRPM2 is functional in dopaminergic (DA) neurons, whole cell current recordings were performed in brain slices obtained from adult mice SNpc region. Both DA and GABAergic neurons are present in the SN region. Thus, we initially identify the DA neurons, as DA and GABAergic neurons exhibit different electrophysiological properties. A hyperpolarizing pulse of −50mV for 1.5s duration was applied to all cells to induce an I_h current. An I_h current ratio was calculated by measuring the current at the end of the capacitive transient over the amplitude of the current at the end of the voltage command. Consistent with previous reports [36], DA neurons, exhibited I_h ratio< 0.6, whereas GABA neurons showed a small I_h (I_h ratio >0.6) (Fig. 2H). More importantly, in DA neurons, bath application of H_2O_2 induced an inward current and the current was inhibited by the addition of ACA (Fig. 2I, J), which is consistent with previous reports [17]. Together, these results indicate that H_2O_2 activates TRPM2 channels and induces Ca^{2+} influx in DA neurons.

MPP^+ facilitates hydrogen peroxide induced Ca^{2+} influx by upregulating TRPM2

Since MPP^+ induces H_2O_2 accumulation and facilitates Ca^{2+} influx, we investigated the relationship of TRPM2 channels and MPP^+. Addition of MPP^+ or MPTP-treatments significantly increased TRPM2 level in *vitro* (Fig 3A) and in *vivo* (Fig 3B) respectively. Also, TRPM2 levels were upregulated in SNpc region of human PD patients (Fig 3C), when

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compared with age matched control human samples. TRPC1 levels is decreased, which is consistent with previous reports [31,6]. Notably, MPTP treatments demonstrated neurodegenerative effects in SNpc by the significant decrease in tyrosine hydroxylase (TH) expression (Fig 3B), a precursor to PD [37]. Intracellular Ca^{2+} studies showed the effect of MPP^+ on Ca^{2+} influx through H_2O_2 inducement, which was also significantly abolished by the addition of FFA (Fig. 3D, E). Moreover, MPP^+ pretreatment significantly facilitated H_2O_2 induced inward Ca^{2+} inward currents without causing changes to the channel's properties. Again, FFA abolished the increased Ca^{2+} influx effect of MPP^+ (Fig. 3F–H). Consistent with these results, treatment mice brain slice with MPP^+ facilitated the inward current that was similar as induced by H_2O_2 (Fig 2I–J), which was abolished by 10μM ACA (Fig. 3I–J). Together, these findings suggest that TRPM2 is upregulated in neurotoxin models of PD that causes intracellular Ca^{2+} overload via the TRPM2 channels.

TRPM2 inhibition prevents cellular death induced by MPP^+ and H_2O_2

To understand the role of TRPM2 in MPP^+ -induced cell death, we examined the effect of FFA in SH-SY5Y cells. Calpain activity is known to be critical for neuronal apoptosis [38]. Exposure to MPP^+ leads to increased calpain activity and FFA treatment significantly prevented calpain activation (Fig. 4A). Furthermore, the decrease in mitochondrial cytochrome *c* levels by MPP^+ was attenuated by FFA (with reciprocal increase in the cytosol) (Fig. 4B). Release of cytochrome *c* into the cytosol activates the caspase cascade of pro teases, which mediates the biochemical and morphological alterations characteristic of apoptosis. Increased expression of the apoptotic protease caspase 3 and pro-apoptotic proteins Bak, Bid and Bad was also observed with MPP^+ treatment and FFA significantly decreased these protein levels (Fig 4C). Furthermore, FFA partially rescued cellular death upon MPP^+ as well as H_2O_2 treatment (Fig. 4D and 4E). Cumulatively, these results suggest that TRPM2 facilitates mitochondrial dysfunction and might play an important role in the cascade of cell death.

Antioxidants rescue cellular death by inhibiting TRPM2

To establish the role of TRPM2 in oxidative stress induced by MPP^+, we next investigated the effects of antioxidant resveratrol (RSV) and N-acetylcysteine (NAC) on TRPM2 channel function. Antioxidants have shown efficient protection against the apoptotic cascade induced by oxidative stress [39,40]. Treatment with RSV or NAC significantly prevented the accumulation of H_2O_2 caused by MPP^+ (data not shown) and therefore abolished the MPP^+ -facilitated Ca^{2+} influx in SH-SY5Y cells (Fig. 5A, B). Under whole cell recording, TRPM2 currents with MPP^+ treatment was also significantly decreased by either RSV or NAC without changing the IV properties (Fig. 5C, D). Notably, consistent with previous reports [39,41], RSV and NAC moderately rescued cell death from MPP^+ treatment (Fig. 5E, F). Together, these results suggest that RSV and NAC down regulate TRPM2 function to prevent Ca^{2+} overload and thus rescue cellular death by relieving oxidative stress.

Knockdown of TRPM2 inhibits MPP^+ induced apoptosis

Although pharmacological inhibition of TRPM2 showed increased cell survival, we used additional methods to confirm that TRPM2 directly contributes to the Ca^{2+} overload and apoptosis induced by MPP^+. Consistent with FFA treatment, silencing TRPM2 significantly

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decreased the levels of TRPM2 proteins and other apoptotic proteins (induced upon MPP^+ treatment) such as: caspase 3, Bak, Bid and Bad (Fig 6A). To have further evidence, immunocytochemical studies showed decrease in the TRPM2 expression in the plasma membrane in siTRPM2 cells pretreated with 250μM of MPP^+ when compared with control siRNA + MPP^+ treated cells (Fig 6B). Silencing TRPM2 also abolished the effect of MPP^+ on H_2O_2-induced increase in Ca^{2+} influx (Fig 6C, D). Similar results were also observed with electrophysiological data, which showed that TRPM2 currents with MPP^+ treatment were significantly decreased by silencing TRPM2 (Fig. 6E, F). Furthermore, silencing TRPM2 rescued cellular death from MPP^+ treatment as shown by MTT assay (Fig. 6G) and PI/ Annexin V staining (Fig. 6H). Finally, the MPP^+ induced caspase 3 activity was also attenuated by TRPM2 silencing (Fig 6I). Taken together, the data presented here indicates that the apoptosis induced by MPP^+ is mediated via TRPM2-specific Ca^{2+} influx.

Overexpression of TRPM2 exacerbates MPP^+ induced cell death

We next evaluated the localization of TRPM2, which was present in the plasma membrane of TH-positive dopaminergic neurons (Fig. 7A). Importantly, MPTP treated mice again showed increase in TRPM2 protein levels (Fig. 7A). To further establish the role of TRPM2 in the loss of dopaminergic cells, we overexpress TRPM2 in differentiated SH-SY5Y cells (Fig 7B). Overexpression TRPM2 significantly increased H_2O_2-induced currents when compared with control cells (Fig. 7C–E). Importantly, treatment with MPP^+ facilitated H_2O_2-induced currents in cells that overexpress TRPM2 (Fig. 7C–E). Moreover, overexpression of TRPM2 not only decreased the cell survival, but also exacerbates MPP^+-induced cell death (Fig. 7F). Cumulatively, these results further confirmed that neurotoxins such as MPP^+ facilitates H_2O_2-induced TRPM2 currents that induce cell death.

Discussion

By using MPP^+, as a model for PD, we have elucidated the role of TRPM2 in modulating the loss of dopaminergic neurons. Previous studies from our lab have shown that MPP^+ induces mitochondrial dysfunction and induces loss of dopaminergic neurons [6]. Here we found that administration of MPP^+ induces accumulation of H_2O_2, leading to cell death by increasing $[Ca^{2+}]_i$ which can be attenuated by the addition of TRPM2 inhibitor ACA and FFA. Ca^{2+} homeostasis plays a central role in neuronal signaling. The alterations in cytoplasmic free Ca^{2+} are important contributory factors for apoptosis and play a major role in the initiation of apoptosis [42,43]. Disturbances in neuronal Ca^{2+} homeostasis have been implicated in variety of neuropathological conditions including PD [44]. Recently, the role of TRPM2 in neurodegenerative diseases has gained much attention in recent years due to its high expression in the brain [13] and its relationship to oxidative stress-induced neuronal death. Notably, burst firing in neurons present in the substantia nigra pars reticulata (SNr) with dopamine depletion has particular implications for PD. In addition overproduction of ROS, from mitochondrial dysfunction and from altered α-synuclein is increased in PD and could contribute to the loss of DA neurons as observed in PD [45,46]. Activation of TRPM2 channels is necessary for burst firing in SNr neurons and their responsiveness to modulatory H_2O_2 [47], suggests that increased glutamatergic input from the STN might interact with

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elevated H_2O_2 to promote burst firing in PD, which may be attributed to increased activation of TRPM2.

TRPM2 channel is expressed in substantia nigra (SN) neurons [47,17] and is tightly regulated by ROS. H_2O_2 can gate the channel directly or indirectly [20,21]. In the indirect pathway, TRPM2 activation is primarily based on its ability to release ADPR from mitochondria [35]. Thus, increased ROS level by the addition of neurotoxins such as MPP^+ may activate TRPM2 channel function and expression. Once inside the neurons, MPP^+ is transported into mitochondria where it interferes with the respiration complex I and inhibits mitochondrial respiration, resulting in the increased formation of superoxide anions [48,2]. An increase in ROS levels is strongly implicated in the pathogenesis of PD, either as a cause or downstream hallmark of dopaminergic degeneration [49,50]. Using MPTP, as an *in vivo* model for PD [51], and PD patient samples we have elucidated that TRPM2 expression is increased under these conditions that could lead to the degeneration of dopaminergic neurons. Importantly, MPP^+ not only increased TRPM2 levels, but also facilitated TRPM2-mediated Ca^{2+} influx and current induced by H_2O_2. These results also support our previous studies in which we have evaluated the role of TRPC1 channels in the neuronal survival [6,31,52]. Consistent with cell culture studies, similar results was observed in DA neurons in SN area.

Our cell culture studies also showed that TRPM2 is involved in the apoptosis in neurons. Apoptosis is induced in neurons by various insults including oxidative stress, metabolic compromise, excitotoxicity, and presence of neurotoxins. Apoptotic signaling is a multistep pathway induced by opening a mitochondrial mega-channel, followed by decline in membrane potential, release of apoptosis-inducing factors, and activation of caspases and calpains [53]. Inhibition of TRPM2 by FFA significantly decreased Ca^{2+} influx, calpain activity, the activation of caspases and consequently showed more resistance to MPP^+-induced cell death. Consistently, TRPM2 knockdown also showed a significant decrease in TRPM2 levels, which resulted in decrease Ca^{2+} influx, decrease activation of caspases and consequently more resistance to MPP^+-induced cell death. In contrast, overexpression of TRPM2 significantly exacerbates MPP^+-induced cell death. Since oxidative stress produced ROS participates in the apoptotic death of dopaminergic neurons, antioxidants may play an important role in preventive and/or complementary therapies for Parkinson's disease. Additionally, there is a report indicating that rotenone, a toxin used to generate animal models of PD, can evoke outward current associated with increases in intracellular Ca^{2+} and increase ROS production, both of which are abolished by N-(p-amylcinnamoyl) anthranilic acid (a TRPM2 channel blocker) [54]. To further demonstrate the role of TRPM2 in oxidative stress induced by MPP^+, we explored the effects of antioxidants resveratrol (RSV) and N-acetylcysteine (NAC) on the TRPM2 channel activation as antioxidants has shown to be efficient protectors against the apoptotic cascade induced by oxidative stress [39,40]. Recent literature has also suggest that antioxidants, such as RSV and NAC, may exert neuroprotective effects [39–41], but its role in neurotoxin models has not been established. Hence our data shows that antioxidants decreased H_2O_2-induced TRPM2 mediated Ca^{2+} influx and inhibited cell death, which confirmed the role of TRPM2 in MPP^+ -induced cell death. In addition a strong correlation between TRPM2 and ROS mediated apoptosis was also observed, indicating that excessive increase in cytosolic Ca^{2+} via the TRPM2 channels

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leads to neuronal loss. In contrast, lack of Ca^{2+} entry via SOCE channels have also been shown to lead to initiate apoptosis [31,6], thus a tight balance in Ca^{2+} homeostasis is important to maintain neuronal survival.

In summary, we have used cell culture, animal models, and patient samples to establish that TRPM2 expression and/or function is increased in neurotoxin models that mimic PD. Our results indicate that TRPM2 is up regulated upon treatment with MPP^+ and MPTP treatment. Moreover, increase of TRPM2 function upon MPTP/MPP^+ treatment lead cells to be more vulnerable to apoptosis, mainly due to increased ROS level, increased activity of calpain, and thereby increases caspase 3 activity, followed by the activation of downstream apoptotic pathway. Importantly, TRPM2 inhibition by FFA or knockdown showed increased protection by preventing MPP^+-mediated ROS increase and inhibiting apoptosis. Taken together our results suggest that TRPM2 expression and function are important for dopaminergic neuronal survival. However, since other Ca^{2+} channels are also involve in apoptosis [6,55,56,42], TRPM2 may not be the sole reason for neuronal death and future research is needed to tease out the details of TRPM2 in dopaminergic neuronal degeneration by neurotoxin MPTP/MPP^+.

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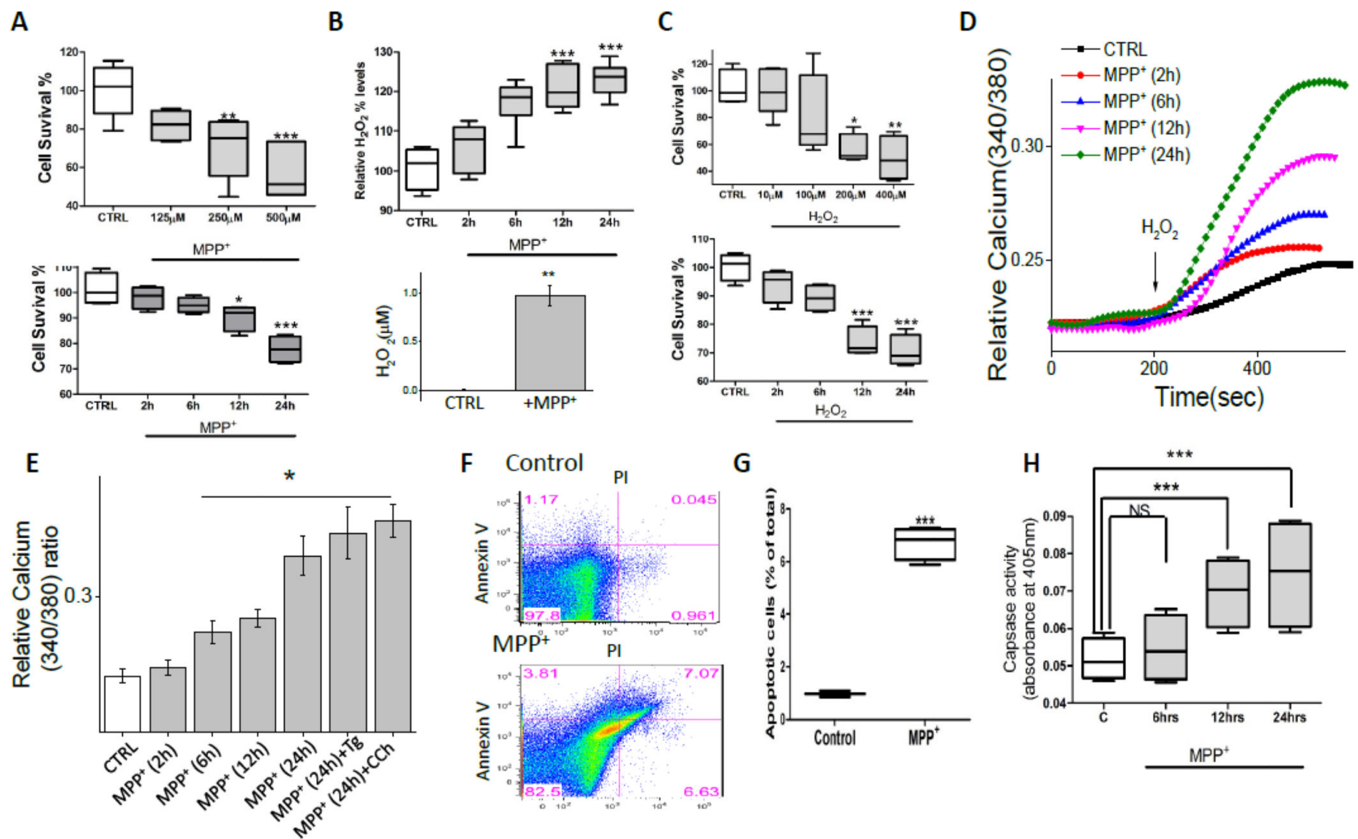

Figure 1. MPP$^+$ induces accumulation of hydrogen peroxide increasing Ca^{2+} level followed neuron death

(A) MTT assays performed on control and MPP+ treatment cells. MPP time and concentration dependent inhibited cells survival. (B) Application of 500μM MPP$^+$ induced a time dependent H_2O_2 accumulation in SH-SY5Y cells. The bottom figure indicated quantification of 500μM MPP$^+$ induced H_2O_2 in 24 hours. (C) Hydrogen peroxide time and concentration dependent inhibited cells survival. (D) Ca^{2+} imaging was showed the 2mM hydrogen peroxide induced Ca^{2+} influx in control and in the presence of 500μM MPP$^+$ in SH-SY5Y cells. Analog plots of the fluorescence ratio (340/380) from an average of 40–60 cells are shown. (E) Quantification (mean ± SD) of fluorescence ratio (340/380) under various conditions. * indicates significance ($p<0.05$) versus control. (F) SH-SY5Y cells were treated with 500 μM MPP$^+$ for 24 h. PI/ Annexin V staining were used to identify apoptotic cells. Representative Flow cytometer plots are shown, which were performed in 3 independent experiments with similar results unless otherwise indicated. The percentage of apoptotic-cell death is as indicated by the total number of cells in the upper right quadrant. (G) Apoptotic cell population is expressed in the right quadrant of the total cell population is represented by the bar diagram. (H) Bar diagram represents the relative Caspase 3 activity was measured using the kit in SH-SY5Y cells pretreated with 500μM MPP$^+$ at the given time points.

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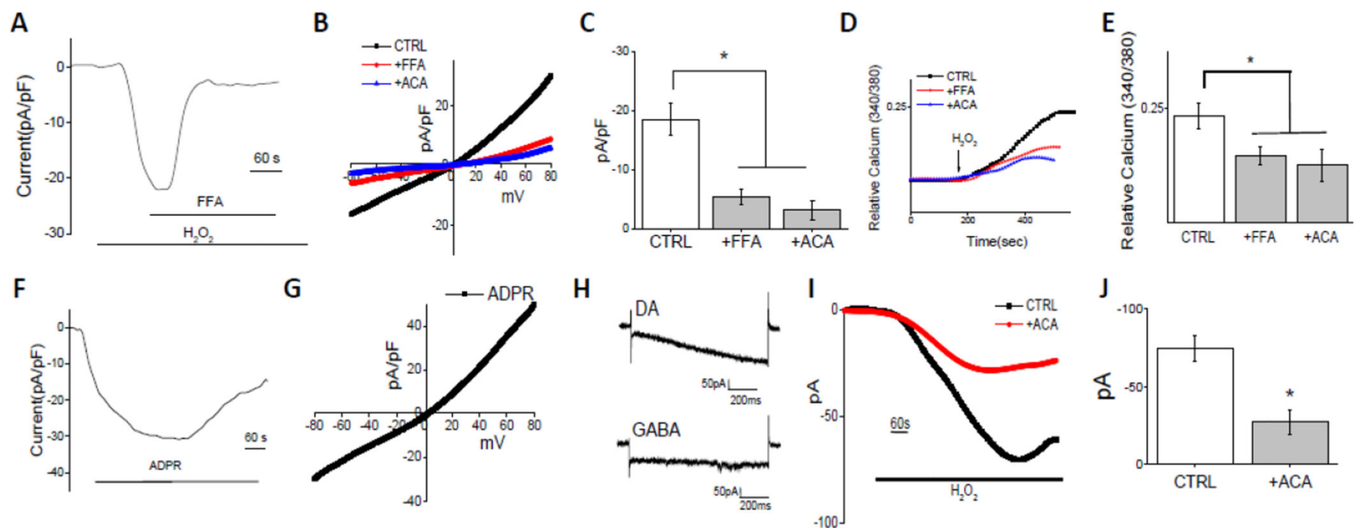

Figure 2. Hydrogen peroxide induces Ca^{2+} influx by TRPM2 channels in SH-SY5Y cells

(A) Application of 2mM hydrogen peroxide induced a none-selectively inward current, which is inhibit by 100μM FFA or 10μM ACA. Average IV curves and current intensity at −80mV under these conditions are shown in (B) and (C). (D) Ca^{2+} imaging was performed in control and in the presence of 100μM FFA or 10μM ACA in SH-SY5Y cells. Analog plots of the fluorescence ratio (340/380) from an average of 40–60 cells are shown. (E) Quantification (mean ± SD) of fluorescence ratio (340/380). (F) Whole cell patch recording showed that application of 1mMM ADP-ribose in the pipette solution induced a none-selectively inward current in SH-SY5Y cells. Average IV curves at −80mV under these conditions are shown in (G). * indicates significance ($p<0.05$) versus control. (H) shows current traces evoked by −50mV steps in mice brain slice. (I) Application of 2mM hydrogen peroxide induced an inward current at −50mV in mice brain slice, which is inhibit by 10μM ACA. Current intensity under these conditions are shown in (J).

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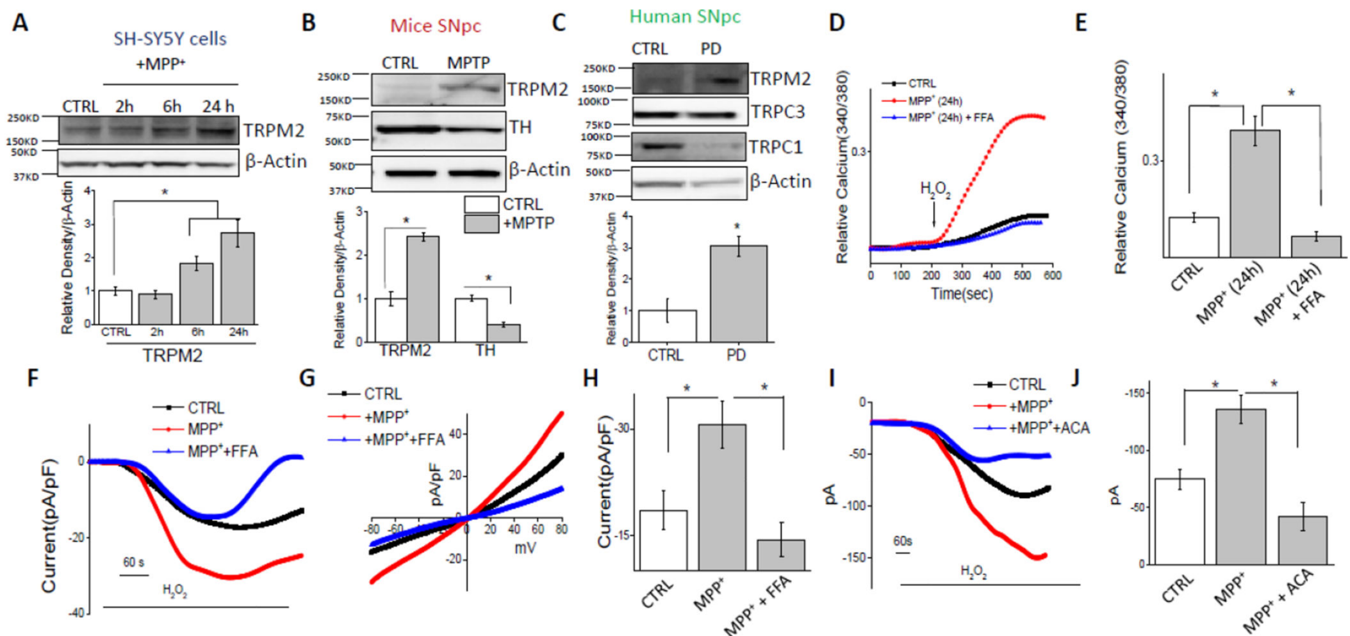

Figure 3. MPP$^+$ facilitates hydrogen peroxide induced Ca^{2+} influx by upregulate TRPM2

(A) SH-SY5Y Cell lysates were resolved and analyzed by western blotting. Antibodies used are labeled and β-actin as used loading control. Western blots of lysates from SN tissues from control and MPTP treatment mice are shown in (B), antibodies used are labeled in the figure. (C) SN tissues from control and PD samples were resolved and analyzed by western blotting. (D) Ca^{2+} imaging was performed in control and various conditions in SH-SY5Y cells. Analog plots of the fluorescence ratio (340/380) from an average of 40–60 cells are shown. (E) Quantification (mean ± SD) of fluorescence ratio (340/380). (F) Application of 2mM hydrogen peroxide induced a none-selectively inward current under various conditions. Average IV curves and current intensity at −80mV under these conditions are shown in (G) and (H). * indicates significance ($p<0.05$) versus control. (I) MPP$^+$ pretreatment 6 h facilitated the inward current induced by hydrogen peroxide(2mM) at −50mV in mice brain slice, which is abolished by 10μM ACA. Current intensity under these conditions are shown in (J).

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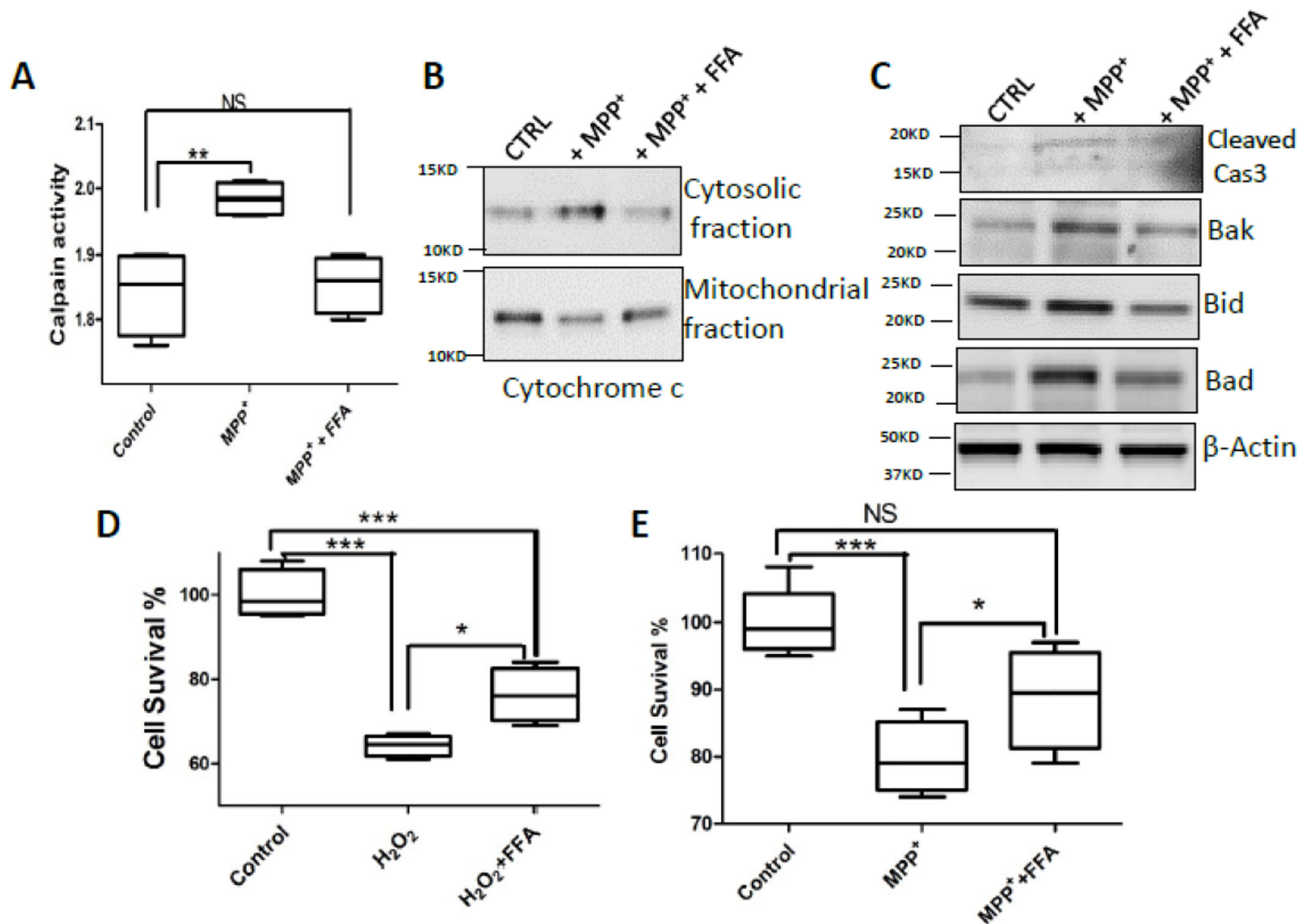

Figure 4. Inhibit TRPM2 rescue cells from death via downregulate apoptosis

(A). Representative bar diagram showing the calpain activity is measured using the kit in SH-SY5Y cells pretreated with 500μM MPP^+ and 50μM FFA respectively for 24hrs. N=3, **, p<0.01. (B) Western blots performed on mitochondrial fraction and cytosolic fraction. (C) SH-SY5Y Cell lysates were resolved and analyzed by western blotting. Antibodies used are labeled and β-actin as used loading control. (D) and (E), Bar diagram showing the cell viability assay (MTT assay) in the SH-SY5Ycells, pretreated with 2mM H_20_2 or 500μM MPP^+ in presence of 50μM FFA respectively. Each bar gives the mean ± SEM of 4 separate experiments. * indicates significance *, p<0.05 **, p<0.01 and *** p<0.001.

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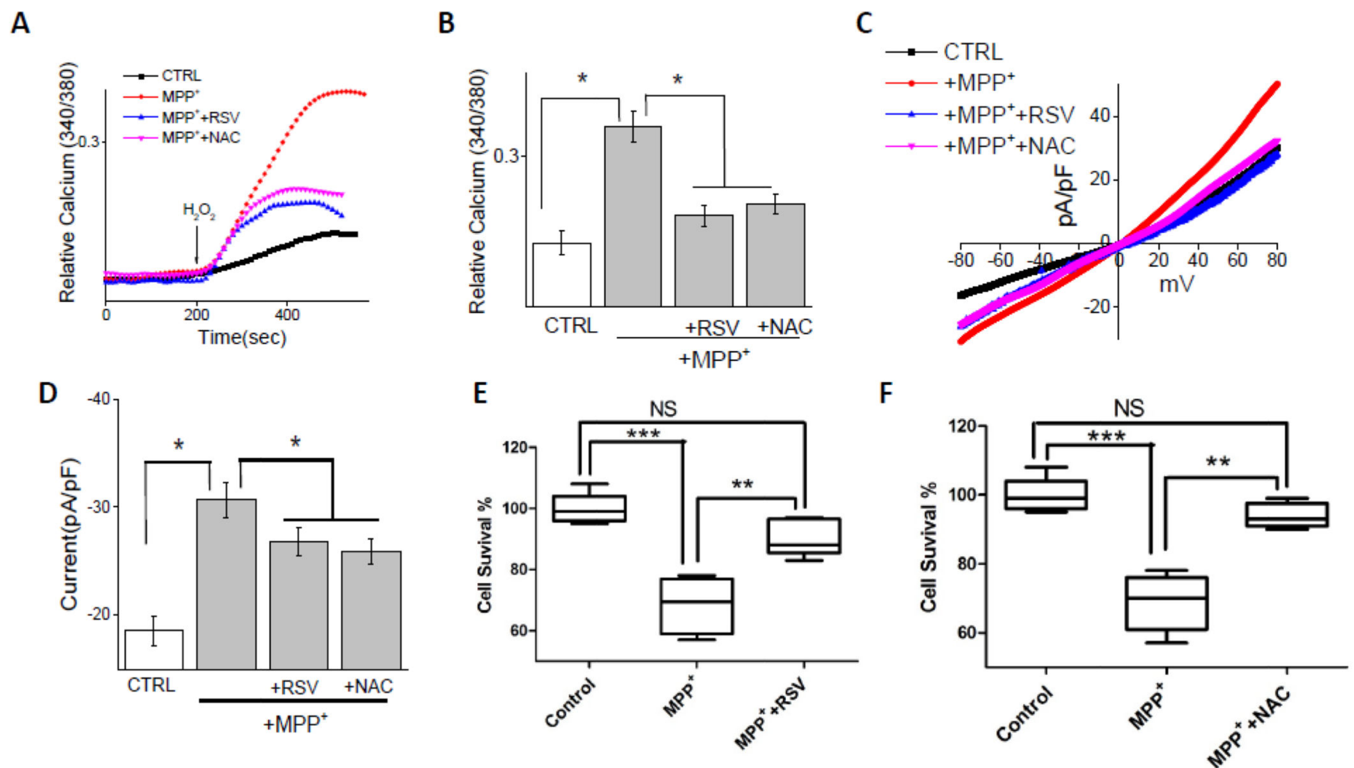

Figure 5. Antioxidant rescue cells death by inhibit TRPM2

(A) Ca^{2+} imaging was performed in control and various conditions in SH-SY5Y cells. Analog plots of the fluorescence ratio (340/380) from an average of 40–60 cells are shown. (B) Quantification (mean ± SD) of fluorescence ratio (340/380). (C) and (D) Whole cell patch recording showed average IV curves and current intensity at −80mV under these conditions. *indicates significance ($p<0.05$). (E) and (F) Bar diagram showing the cell viability assay (MTT assay) in the SH-SY5Ycells, pretreated with 500μM MPP^{+} in presence of 25μM RSV or 100μM N-ace respectively. Each bar gives the mean ± SEM of 4 separate experiments. NS indicates no significance and * indicates significance *, $p<0.05$ **, $p<0.01$ and *** $p<0.001$.

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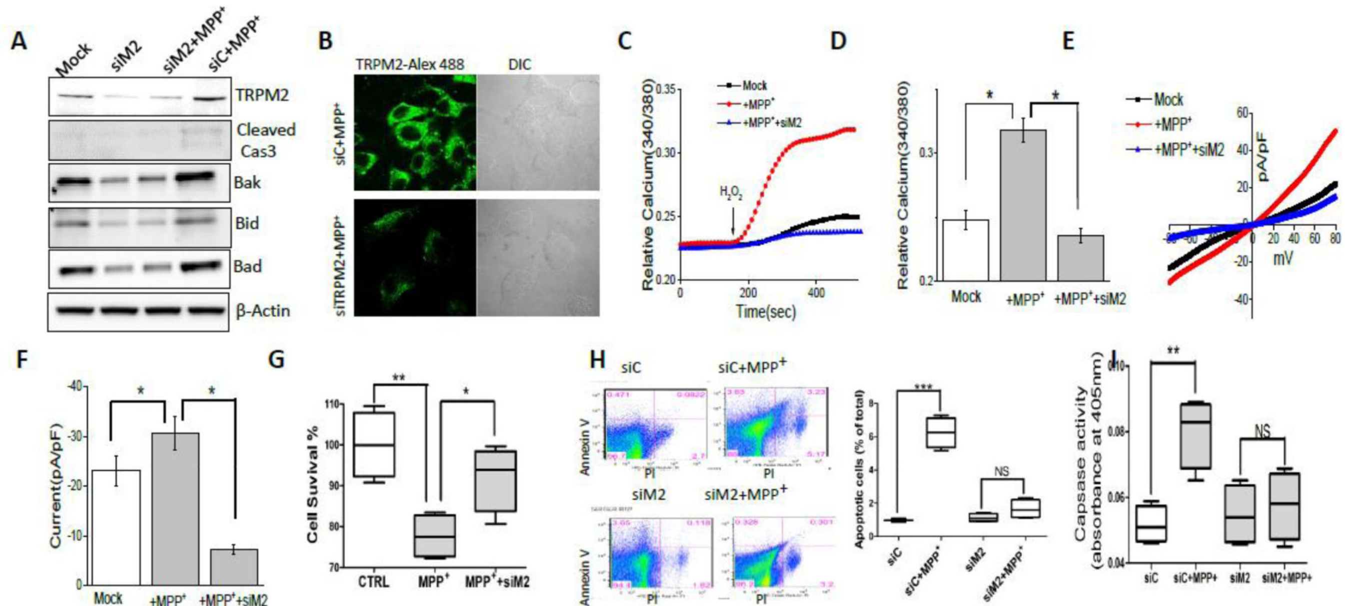

Figure 6. Knockdown TRPM2 inhibited MPP$^+$ induced apoptosis

(A) SH-SY5Y cell lysates were resolved and analyzed by western blotting. Antibodies used are labeled and β-actin as used loading control. (B) Confocal images showing the localization of endogenous TRPM2 protein in SHSHY-5Y siC and siTRPM2 cells pretreated with 500μM MPP$^+$ for 24 hours. (C) Ca^{2+} imaging was performed in control and various conditions in SH-SY5Y cells. Analog plots of the fluorescence ratio (340/380) from an average of 40–60 cells are shown. (D) Quantification (mean ± SD) of fluorescence ratio (340/380). (E) and (F) Whole cell patch recording showed average IV curves and current intensity at −80mV under these conditions. (G) MTT assays under various conditions in SH-SY5Y cells. *indicates significance ($p<0.05$). (H) The siRNA TRPM2 knockdown SH-SY5Y cells and siRNA control cells were treated with 500 μM MPP$^+$ for 24 h. PI/ Annexin V staining were used to identify apoptotic and necrotic cells. Representative Flow cytometer plots are shown; stains were performed in 3 independent experiments with similar results. The percentage of apoptotic-cell death is as indicated by the total number of cells in the upper right quadrant. Apoptotic cell population is expressed in the right quadrant of the total cell population is represented by the bar diagram. (I). Bar diagram represents the relative Caspase 3 activity was measured using the kit in siRNA TRPM2 knockdown SH-SY5Y cells and control cells pretreated with 500μM MPP$^+$ for 24hrs. Each bar gives the mean ± SEM of 4 independent experiments.

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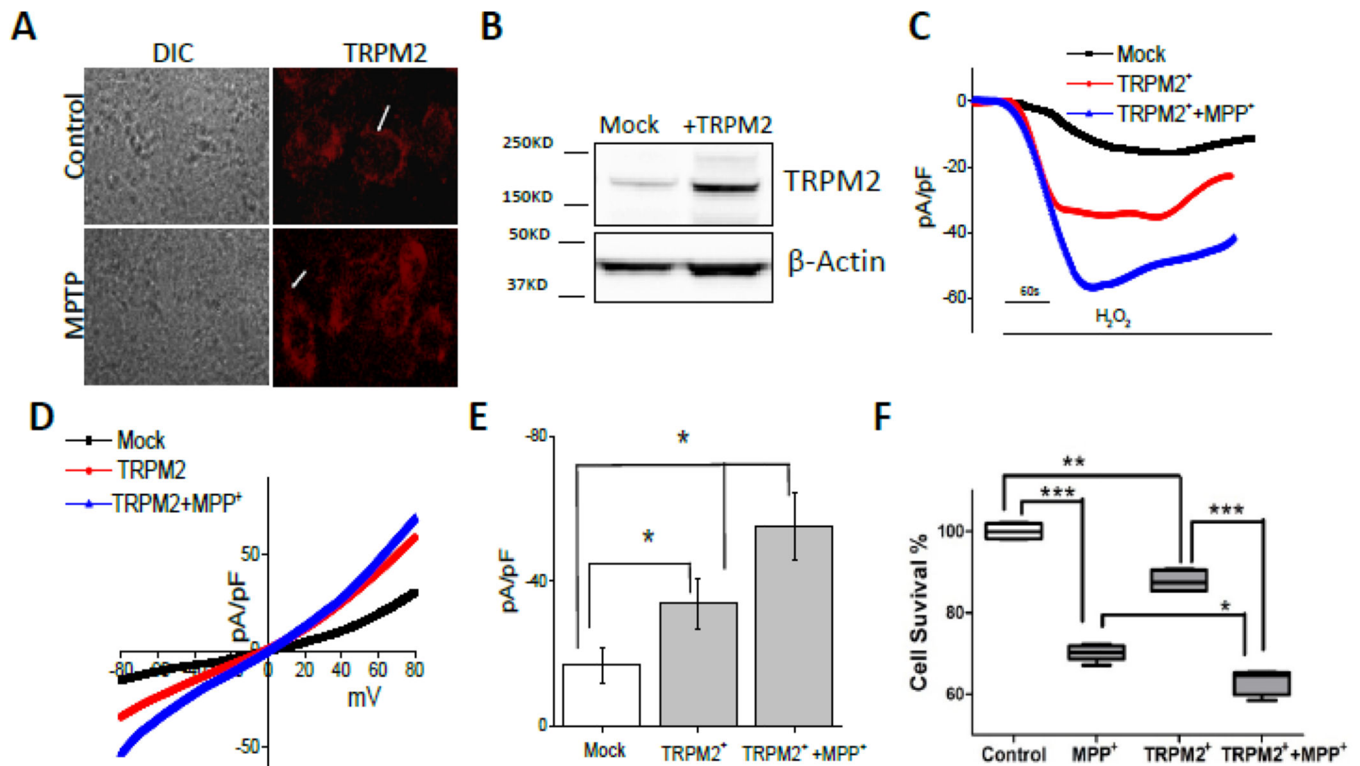

Figure 7. Overexpression TRPM2 exacerbate MPP^+ induced cell death

(A) Confocal images showing localization of endogenous TRPM2 in dopaminergic neurons in the SNpc region. (B) SH-SY5Y cell lysates from TRPM2 overexpression and mock were resolved and analyzed by western blotting. Antibodies used are labeled and β-actin as used loading control. (C) Whole cell patch recording showed application of 2mM hydrogen peroxide induced none-selectively inward currents under various conditions. Average IV curves and current intensity at −80mV under these conditions are shown in (D) and (E). (F) MTT assays under various conditions in SH-SY5Y cells. *indicates significance ($p<0.05$).