α-Synuclein up-regulation and aggregation during MPP⁺-induced apoptosis in neuroblastoma cells: Intermediacy of transferrin receptor iron and hydrogen peroxide.

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Running title: Role of transferrin iron in α-synuclein up-regulation and apoptosis.

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Key words: caspase-3, α-synuclein, transferrin receptor, iron, oxidative stress, glutathione peroxidase, Parkinson’s disease.

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Abbreviations: MPP⁺, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; Carboxy-H2DCFDA, carboxy-2',7'-dichlorodihydrofluorescein diacetate; FeTBAP, Fe(III) tetrakis (4-benzoic acid) porphyrin; L-NAME, L-nitroarginine-N-methyl ester; ROS, reactive oxygen species; DPBS, Dulbecco’s phosphate buffered saline; HBED, N, N'-bis (2-hydroxybenzyl) ethylenediamine-N, N'-diacetic acid; TfR, transferrin receptor; α-syn, α-synuclein; AS α-syn, antisense α-synuclein; PD, Parkinson’s disease.
Abstract

1-Methyl-4-phenylpyridinium (MPP⁺) is a neurotoxin that causes Parkinson’s disease in experimental animals and humans. Despite the fact that intracellular iron was shown to be crucial for MPP⁺-induced apoptotic cell death, the molecular mechanisms for the iron requirement remain unclear. We investigated the role of transferrin receptor (TfR) and iron in modulating the expression of α-synuclein (α-syn) in MPP⁺-induced oxidative stress and apoptosis. Results show that MPP⁺ inhibits mitochondrial complex-1 and aconitase activities leading to enhanced H₂O₂ generation, TfR expression and α-syn expression / aggregation. Pretreatment with cell-permeable iron chelators, TfR-antibody (that inhibits TfR-mediated iron uptake), or transfection with glutathione peroxidase (GPx1) enzyme inhibit intracellular oxidant generation, α-syn expression / aggregation, and apoptotic signaling as measured by caspase-3 activation. Cells over-expressing α-syn exacerbated MPP⁺ toxicity whereas antisense α-syn treatment totally abrogated MPP⁺-induced apoptosis in neuroblastoma cells without affecting oxidant generation. The increased cytotoxic effects of α-syn in MPP⁺-treated cells were attributed to inhibition of mitogen-activated protein kinase (MAPK) and proteasomal function. We conclude that MPP⁺ induced iron-signaling is responsible for intracellular oxidant generation, α-syn expression, proteasomal dysfunction, and apoptosis. Relevance to Parkinson’s disease is discussed.
Introduction

Parkinson’s disease (PD) is one of the major human neurodegenerative disorders that is characterized by a progressive loss of dopaminergic neurons and the formation of Lewy body aggregates (1,2). Increasing evidence suggests that α-synuclein (α-syn), parkin, ubiquitin and iron constitute a major fraction of Lewy body aggregates (3). Defects in mitochondrial complex-1 activity and dopamine deficiency were detected in patients suffering from PD (4,5). Mitochondrial complex-1 inhibitors such as 1-methyl-4-phenylpyridinium (MPP+), a metabolic product of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and rotenone have been shown to induce symptoms similar to those of PD in experimental animals and humans (6,7). Several in vitro studies using MPP+ and rotenone showed that these compounds can induce oxidative stress and apoptosis and other biochemical changes similar to those observed in patients afflicted with idiopathic PD (8,9). The conversion of MPTP to MPP+ is required for toxicity and this conversion is catalyzed by monoamine oxidase type B in the brain (10). MPP+ is transported into dopaminergic neurons by the dopamine transporter and is concentrated into mitochondria where it causes ATP depletion by inhibiting complex-1 activity, leading to alterations in the mitochondrial membrane potential and finally to cell death (11).

Recent studies show that MPTP and/or MPP+ and rotenone induce α-syn expression and its aggregation (12,13). α-Syn aggregation is considered to be one of the pathological hallmarks of PD (14,15). However, the role of α-syn in cellular toxicity is not well understood. In some cells, α-syn over-expression was found to be protective whereas in others α-syn over-expression elicited increased toxicity (16,17), but the
general consensus is that α-syn over-expression is ultimately toxic to dopaminergic neurons (18). However, the mutant forms of α-syn are more toxic compared to the wild type α-syn (19,20). Additional studies elucidating the molecular signaling events that lead to the up-regulation and aggregation of α-syn in cells are, therefore, warranted.

Reports indicate that MPP⁺ promotes the aggregation of α-syn in neuroblastoma cells (21). Iron and H₂O₂ exacerbated α-syn aggregation in vitro (22). Previously, we showed that MPP⁺ up-regulates transferrin receptor (TfR) expression and iron uptake resulting in oxidative stress and apoptosis (23). In the present study, we sought to determine whether MPP⁺-induced TfR-iron plays a critical role in the up-regulation/aggregation of α-syn leading to enhanced apoptosis in human neuroblastoma cells.
Materials and Methods

Materials — Carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H$_2$DCFDA) was purchased from Molecular Probes Inc, USA. Fe(III) tetrakis (4-benoic acid) porphyrin (FeTBAP) was synthesized according to the published procedure (24). Hydroxybenzyl ethylenediamine (HBED) was obtained as a gift from Dr. Cherakuri Muralikrishna (National Institutes of Health). 1-Methyl-4-phenylpyridinium (MPP$^+$) iodide, L-nitroarginine-N-methyl ester (L-NAME) and other chemicals for buffer preparations were purchased from Sigma Chemical Co, USA. Lipofectamine reagent was obtained from Invitrogen Inc, USA. Adenovirus containing full length GPx1 (Ad.CMV-GPx1) was a generous gift from Dr. Larry Oberley (University of Iowa, USA). Ad-CMV-GFP was obtained from the adenoviral core facility, Medical College of Wisconsin, Milwaukee. MAP kinase inhibitor, U0126 was obtained from Calbiochem Inc. Monoclonal antibody, 42/6, against human TfR was a gift from Dr. Ian Towbridge (Salk Institute, San Diego, CA).

Culturing of human neuroblastoma cells — Human neuroblastoma cells (SH-SY5Y) obtained from American Type Cell Collection were transferred to 75-cm$^2$ filter vent flasks (Costar, Cambridge, MA), grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS), L-glutamine (4 mmol/liter), penicillin (100 units/ml), and streptomycin (100 µg/ml), and incubated at 37 °C in a humidified atmosphere of 5% CO$_2$ and 95% air. For experiments, cells were seeded in six well dishes and grown to a confluence of 70-80%. Twelve hours before the start of treatment, medium was replaced with Dulbecco’s modified Eagle’s medium (DMEM) containing
2% FBS. The above conditions were applied to all of the experiments performed in this study.

**Cell treatments** — Unless otherwise stated, all experiments were performed in neuroblastoma cells cultured in 6 well plates. Cells were pretreated for 2 h individually with the following agents: media alone, FeTBAP (25 µM) or L-NAME (2.5 mM), HBED (10 µM), U0126 (25 µM) and caspase inhibitors (5 µM) followed by the addition of MPP⁺ to a final concentration of 5 mM. Following a 16 h incubation period, cell culture media were aspirated and cells were washed once with Dulbecco’s phosphate buffered saline (DPBS) and collected by gentle scraping. The cell pellets were washed twice with DPBS and used for subsequent assays. For the time course experiments, neuroblastoma cells were treated with 5 mM MPP⁺ and cells were collected by gentle scraping at different time points. In some experiments, neuroblastoma cells were treated with 150 µM H₂O₂ for 6 h.

**Caspase-3 activity** — Caspase-3 activity was determined in neuroblastoma cell lysates using the Apo-Alert Kit (Clontech, Palo Alto, CA). After collection, cells were suspended in 100 µl of lysis buffer supplied in the kit and passed through a 24 gauge needle 10 times to ensure complete lysis. The lysate was centrifuged at 4°C for 10 min at 10,000 rpm and 50 µl of the clear supernatant was used for the activity assay according to the manufacturer’s protocol. The increase in the optical density at 405 nm was considered as an index of caspase-3 activity.

**Aconitase activity** — Neuroblastoma cells were washed twice with cold DPBS and lysed with buffer containing 0.2% Triton-X-100, 100 µM DTPA and 5 mM citrate in PBS. The activity of aconitase was measured in 100 mM Tris-HCl (pH 8.0) containing
20 mM D,L-trisodium isocitrate at 37° C. An extinction coefficient for cis-aconitate of 3.6 mM at 240 nm was used (25).

**Complex-1 activity** – The activity of mitochondrial complex I was measured as described previously (26). Mitochondrial pellets were frozen and thawed three times in hypotonic buffer (20 mM potassium phosphate buffer pH 7.2). Fifty microliters of the supernatant was added to 1 ml of potassium phosphate buffer (20 mM, pH 7.2) containing NADH (100 µM), 65 µM coenzyme Q1, 5 mM MgCl₂, 2 mM KCN, 2.5 mg / ml bovine serum albumin and 2 µg / ml antimycin A in a 1.5 ml cuvette at 37° C. The difference in the rate of NADH oxidation 340 nm was used as an index of complex-1 activity. Under these conditions, the complex-1 specific activity was inhibited by 2 µg / ml rotenone.

**Over-expression of GPx1** – Adenoviral infection with GPx1 in neuroblastoma cells was performed in a serum free medium for 1 h at a multiplicity of infection (MOI) of 500 particles / cell (27), followed by the addition of an equal volume of fresh medium containing 20% FBS and 50 nM sodium selenite. Incubation was continued for 24 h. The medium was replaced 24 h after infection with a medium containing 2% FBS and the cells were subsequently treated with MPP⁺. These conditions produced nearly 100% transfection with recombinant adenovirus, as assessed with an Ad.CMV-GFP reporter gene expression. GPx1 activity was measured as described earlier (27).

**Determination of α-syn and TfR levels** – After terminating the experiment, neuroblastoma cells were washed with ice-cold DPBS and resuspended in 100 µl of RIPA buffer (20 mM Tris-HCl, pH 7.4, 2.5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 1% SDS, 100 mM NaCl, 100 mM sodium fluoride). To a 10 ml solution of the above, the following agents were added: 1 mM sodium vanadate, 10 µg/ml aprotinin,
10 µg/ml leupeptin, and 10 µg/ml pepstatin inhibitors. The cells were homogenized by passing the suspension through a 25-gauge needle (10 strokes). The lysate was centrifuged at 12,000 × g for 10 min and the supernatant was used for analysis. Protein concentrations were determined by the Lowry method (Biorad), and 20 µg was used for the Western blot analysis. Proteins were resolved on a 10% SDS-polyacrylamide gel and blotted onto nitrocellulose membranes. Membranes were washed with Tris-buffered saline (140 mM NaCl, 50 mM Tris-HCl, pH 7.2) containing 0.1% Tween 20 and 5% skim milk to block the non-specific protein binding. Membranes were incubated either with monoclonal antibodies raised against human α-syn (Transduction Laboratories) or a mouse anti-human TfR monoclonal antibody (Zymed Laboratories Inc., San Francisco, CA) (1 µg/ml) in Tris-buffered saline containing 0.1% Tween 20 for 2 h at room temperature), washed five times, and then incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG (1:5000) for 1.5 h at room temperature. Both α-syn and TfR bands were detected using the ECL method (Amersham Biosciences). Statistical significance was obtained using Student’s t-test employing the Sigmastat software.

**RT-PCR analysis** - Following the termination of experiments, the medium was aspirated and 1 ml of TRizol reagent (Invitroge n) was added to cells in 6 well plates and total RNA was extracted using the manufacturer’s protocol (Invitrogen). 5 µg of RNA was used for the first strand cDNA synthesis using first strand cDNA synthesis kit (Amersham) with random hexamers provided in the kit in a final volume of 15 µl. 4 µl of the cDNA mixture was used to amplify mRNA for α-syn, TfR and 18s ribosomal RNA by PCR (as a loading control) using the gene specific primers designed by the Genetics Computer Group, Inc. (GCG) software.
Cloning of sense and antisense α-syn - Total RNA was isolated from neuroblastoma cells using a TRizol reagent (Invitrogen) according to the manufacturer’s protocol. 5 µg of total RNA was used to amplify full length α-syn gene using high fidelity PCR supermix (Hi FI PCR supermix, Invitrogen) using the forward (5’-AAGCT TAGGAATTCCATTAGCCATGGATGTATTC-3’) and reverse (5’-CTCGAG AGATA TTTCTTAGGCTTCAGGTTCGTAGT-3’) primers containing Hind III and XhoI restriction sites (shown in bold and underlined). Following PCR, the amplified product was digested with Hind III and Xho I and ligated into pcDNA3.1 (+) that was pre-digested with the same restriction enzymes in order to obtain the plasmid for over-expressing α-syn (pcDNA3α-syn). The same full length PCR product was cloned into pcDNA3.1 (-) to obtain the plasmid that expresses antisense mRNA for α-syn (AS-syn). The cloned products were confirmed by the DNA sequence of the plasmid.

Transfection of sense and antisense α-syn in neuroblastoma cells – Neuroblastoma cells in 6-well plates (~70% confluent) were transfected individually with 3 µg of pcDNA 3 plasmid containing the sense and antisense α-syn gene using the lipofectamine reagent as described by the manufacturer’s protocol (Invitrogen). Briefly, cells were treated with DNA-lipofectamine complex in 1 ml of DMEM (without FBS and antibiotics) for 4 hours, followed by the addition of equal amounts of complete DMEM (with 10% FBS, pencillin and streptomycin) and cells were further incubated for 24 h. After replacing with a fresh medium, cells were allowed to recover for an additional 24 h and used for the experiments. Under these conditions, the transfection efficiency was found to be nearly 50% using the GFP reporter plasmid (pE GFP-N1, Clontech).
**Measurement of oxidative stress** - The determination of intracellular oxidant production was based on the oxidation of carboxy-2′,7′-dichlorodihydrofluorescein-di-acetate (carboxy-H$_2$DCFDA) to the fluorescent product, carboxy-2′,7′-dichlorofluorescein (carboxy-DCF) (28). Following treatment of cells with MPP$^+$, the media were aspirated, and cells were washed twice with DPBS and then placed in 1 ml of cell culture medium without FBS. The carboxy-H$_2$DCFDA was added to a final concentration of 10 µM and cells were incubated for 20 min. The cells were again washed once with DPBS and maintained in a 1 ml of culture medium. Intracellular fluorescence was monitored at wavelengths of 480/30 (excitation) and 535/40 (emission). The fluorescence intensity values from three different fields of view were calculated using the Metamorph software and averaged.

**Measurement of 26s proteasomal activity** - Proteasome function was measured as reported (29). Briefly, cells were washed with buffer I (50 mM Tris, pH 7.4, 2 mM DTT, 5 mM MgCl$_2$, 2 mM ATP) then homogenized with buffer I containing 250 mM sucrose. Twenty µg of 10,000 X g supernatant were diluted with buffer I to a final volume of 900 µl. The fluorogenic proteasome substrate Z-Leu-Leu-Lys-AMC (trypsin-like) was added in a final concentration of 80 µM. Proteolytic activity was measured by monitoring the release of the fluorescent group 7-amido-4-methylcoumarin (excitation and emission at 380/460 nm).
RESULTS

MPP⁺ induces α-syn over-expression and aggregation in neuroblastoma cells - The addition of MPP⁺ to neuroblastoma cells induced a time-dependent increase in α-syn protein levels (Fig. 1A, monomer). Nearly a three-fold increase in protein expression was detected after a 8h time-period in MPP⁺-treated cells that remained elevated up to 12h (Fig. 1B). During the same time period, both dimeric and oligomeric forms of α-syn were induced (Fig. 1A, monomer and oligomer bands). RT-PCR data also confirmed the same trend as evidenced by the increase in α-syn mRNA levels following MPP⁺ treatment. The transcription of α-syn mRNA started to increase as early as 6 h after MPP⁺ treatment (Fig. 1C). Figure 1C also shows that this observed increase is not due to differences in sample loading, as detected using 18S rRNA during the same time-period. These results indicate that α-syn expression is regulated at both transcriptional and translational levels in response to MPP⁺ treatment. Concomitant with an increased α-syn expression (Fig. 1A, monomer) both dimeric and oligomeric forms of α-syn increased in MPP⁺-treated cells (Fig. 1A).

MPP⁺ induces oxidative stress, TfR expression and apoptosis- The inactivation of aconitase in MPP⁺-treated neuroblastoma cells was measured as an index of oxidative stress. There was a significant decline with time in the aconitase activity after a 4h treatment in cells treated with MPP⁺ (Fig. 2A). During the same incubation period, an increase in the expression of TfR levels was observed (Fig. 2B). As shown, TfR levels increased in MPP⁺-treated cells after 4h and remained elevated up to 12 h (Fig. 2B). The RT-PCR data obtained under these conditions did not show any significant increase in
TfR mRNA levels (Fig. 2C). A significant increase in the caspase 3-like proteolytic activity was detected in neuroblastoma cells treated with MPP⁺. A four to five-fold increase in caspase-3 activity was seen after a period of 12 to 16 h in MPP⁺-treated cells (Fig. 2D). These results demonstrate that MPP⁺ initially causes oxidative stress as demonstrated by the decrease in aconitase activity followed by TfR expression and finally leads to apoptosis. These findings implicate an oxidative mechanism, possibly involving iron, in MPP⁺-dependent apoptosis in neuroblastoma cells.

**Effect of iron chelators and antioxidants on MPP⁺-induced α-syn expression, oxidative stress and apoptosis**—Previously, we reported that MPP⁺ induces intracellular H₂O₂ and iron release in neuroblastoma cells (23). Thus, we surmised that H₂O₂ and iron could be responsible for MPP⁺-induced over-expression and aggregation of α-syn. In control experiments, MPP⁺ treatment caused greater than 50% decrease in the aconitase activity in cells following a 4-6 h treatment with MPP⁺ (Fig. 3A). Pretreatment of neuroblastoma cells with FeTBAP (an SOD mimetic that is also a scavenger of H₂O₂) or a cell-permeable iron chelator, HBED, restored the aconitase activity in neuroblastoma cells treated with MPP⁺ (Fig. 3A). Over-expression of cells with GPx1 that detoxifies intracellular H₂O₂ counteracted MPP⁺-induced inactivation of aconitase (Fig. 3A). Treatment of cells with L-NAME, a non-specific inhibitor of nitric oxide synthase, had little or no effect on oxidative inactivation of aconitase in MPP⁺-treated cells (Fig. 3A). These results suggest that MPP⁺-induced inactivation of aconitase was mediated by intracellular hydrogen peroxide and iron. To investigate more fully the mechanism of iron-induced α-syn aggregation and the origin of intracellular iron, we determined the
effect of a TfR antibody (which binds to the extracellular domain of TfR and inhibits entry of iron into cells). Pretreatment of cells with TfR antibody dramatically inhibited α-syn expression and aggregation (Fig. 3B and C), as well as the transcription of α-syn mRNA (Fig. 3D), suggesting that iron transported through TfR is responsible for α-syn expression and aggregation. The MPP⁺-induced apoptosis, as measured by the proteolytic activation of caspase-3 was inhibited by FeTBAP, TfR ab, and GPx1 over-expression (Fig. 3E). Collectively, these findings implicate a causal role for Tf-iron and H₂O₂ in MPP⁺-induced α-syn up-regulation, aggregation, and apoptosis.

Next we sought to determine whether hydrogen peroxide treatment alone could enhance α-syn expression/aggregation and apoptosis in neuroblastoma cells. Immunoblotting of α-syn revealed that the protein levels were significantly increased in cells exposed to H₂O₂ for 4 h (Fig. 4A and B). The RT-PCR analysis demonstrated an increase in α-syn mRNA during the same incubation time (Fig. 4C). Concomitant with increased expression of α-syn, the caspase-3 activity was also enhanced in cells treated with H₂O₂ (Fig. 4D).

**Effect of modulating α-syn levels on MPP⁺-induced oxidative stress and apoptosis-**

To determine whether α-syn is causally responsible for MPP⁺-dependent apoptosis or whether α-syn up-regulation by MPP⁺ is simply a parallel consequence, we employed α-syn over-expressing and under-expressing neuroblastoma cells. To this end, neuroblastoma cells were individually transfected with 3 µg of pcDNA 3 plasmid containing the sense and antisense α-syn gene, using the lipofectamine reagent as described under *Materials and Methods*. The transfection efficiency was nearly 50%
using the GFP reporter plasmid (pE GFP-N1, Clontech). As shown in Figure 5A, α-syn protein levels in the cytosol increased six-fold in neuroblastoma cells transfected with sense plasmid, and three-fold less than the control in the antisense α-syn-treated cells. We measured caspase-3 activation in these cells after treatment with MPP\(^+\) for 12 h. As shown in Fig. 5B, the caspase-3 activation induced by MPP\(^+\) was completely inhibited in antisense α-syn-treated cells. In contrast, the addition of MPP\(^+\) to α-syn over-expressing cells enhanced the caspase 3 activity compared to control cells (Fig. 5B). These results indicate that α-syn is necessary for MPP\(^+\)-mediated apoptosis.

Next we measured the oxidative stress response in α-syn over-expressing and under-expressing cells. Figures 5C and D show that the aconitase and mitochondrial complex-1 activities were decreased by 2-3 fold in cells treated with MPP\(^+\) for 12 h. However, unlike caspase-3 activation, the aconitase and mitochondrial complex-1 activities remained unchanged in response to modulation of α-syn levels in neuroblastoma cells. MPP\(^+\) treatment of α-syn over-expressing and under-expressing cells did not affect aconitase or complex-1 activities (Fig. 5C and D).

Previously, we reported that MPP\(^+\)-induced oxidation of DCFH to DCF is dependent on intracellular H\(_2\)O\(_2\) and iron (23). MPP\(^+\)-induced DCF fluorescence was inhibited by GPx1 over-expression, cell-permeable iron chelators, and by TfR antibody, suggesting that both TfR-induced iron uptake and intracellular H\(_2\)O\(_2\) were responsible for increased intracellular DCF fluorescence. DCF fluorescence was unaffected in antisense α-syn transfected neuroblastoma cells (Fig. 5E and F). As shown, MPP\(^+\)-induced DCF staining was not inhibited by either α-syn over-expression or transfection with antisense α-syn plasmid. Based on these results, we conclude that MPP\(^+\)-induced oxidative stress is
upstream of α-syn expression and not *vice versa*. α-Syn over-expression in cells may induce apoptotic signaling by mechanisms other than intracellular oxidative stress.

**Possible role of MAP kinase and proteasome in α-syn-mediated apoptosis**—Recent reports suggest that α-syn can inhibit the activities of MAP kinases and proteasome (30,31). This implicates that MPP⁺ induced α-syn might play an indirect role by inhibiting ERK/MAP kinase, as has been suggested previously (30) and that the antisense α-syn-treated cells confer protection against MPP⁺-induced apoptosis, possibly through elevating the MAP kinase activity. Consistent with this interpretation, we found that treatment of cells with a specific ERK/MAP kinase inhibitor, U0126, abrogated the protection afforded by the antisense α-syn towards MPP⁺-induced apoptosis (Fig. 6A). Results also showed that trypsin-like activity associated with the 26S proteasome was diminished in MPP⁺-treated cells and in α-syn over-expressing cells (Fig. 6B); however, the antisense α-syn treatment significantly restored the trypsin-like activity in presence of MPP⁺. These results indicate that α-syn can potentially interfere with ERK/MAP kinase regulation and/or inhibit proteasomal function, i.e., factors leading to enhanced apoptosis in neuroblastoma cells. However it is not yet clear whether the two pathways are mutually exclusive.
DISCUSSION

In this study, we showed that MPP\(^+\), an inhibitor of mitochondrial complex 1, induces Tf-iron dependent oxidative stress, transcriptional up-regulation of \(\alpha\)-syn, protein aggregation and apoptosis in neuroblastoma cells. Pretreatment with anti-TfR antibody, cell-permeable iron chelators, or GPx1 over-expression greatly mitigated Tf-iron uptake, oxidative stress, \(\alpha\)-syn expression, and apoptosis in cells treated with MPP\(^+\). MPP\(^+\)-induced apoptosis was vastly reduced in neuroblastoma cells under-expressing \(\alpha\)-syn and significantly elevated in \(\alpha\)-syn over-expressing cells. The toxic effects of \(\alpha\)-syn could be due to the inhibition of MAP kinase and proteasomal dysfunction. Collectively, these results implicate a critical role for \(\alpha\)-syn in MPP\(^+\)-induced neuronal apoptosis (Scheme 1).

**MPP\(^+\)**-induced oxidative stress and iron signaling on \(\alpha\)-syn induction / aggregation — Protein aggregation is linked to the pathophysiology of neurodegenerative diseases (32). It has been reported that MPP\(^+\), an active metabolite of the neurotoxin, MPTP, promotes \(\alpha\)-syn expression in neuroblastoma cells (12,13). Consistent with the earlier observations, we found that MPP\(^+\) induced \(\alpha\)-syn expression and aggregation in a time dependent manner (Fig. 1 A and B). In addition, the observed increase in \(\alpha\)-syn expression is not only due to its decreased turnover, as previously suggested (33) but presumably occurs even at the transcriptional level, as \(\alpha\)-syn mRNA also increased with time in response to MPP\(^+\) treatment (Fig. 1C). The presence of aggregated \(\alpha\)-syn in the brain is a pathological hallmark of Parkinson’s disease (34). Iron levels have been reported to be elevated in the substantia nigra of PD patients (35). Iron
and H$_2$O$_2$ exacerbated $\alpha$-syn aggregation \textit{in vitro} (22). We have recently shown that MPP$^+$ generates H$_2$O$_2$ in neuroblastoma cells via a mechanism involving the inhibition of a mitochondrial electron transfer enzyme, complex-1 (23). Blocking the electron leakage from mitochondrial NADH dehydrogenase leads to an increase in superoxide generation. The rapid reaction between mitochondrial superoxide and 4Fe-4S centers of mitochondrial complex I and aconitase is well-established (36). During this reaction, the [4Fe-4S]$^{2+}$ is quantitatively oxidized to the [3Fe-4S]$^{1+}$ cluster by superoxide, releasing Fe$^{2+}$ and H$_2$O$_2$. This facilitates the formation of potent oxidants, hydroxyl radical or perferryl iron in mitochondria.

The role of oxidant-induced iron-signaling is becoming increasingly relevant in neurodegenerative diseases (37,38). Mitochondrial toxins that stimulate O$_2^-$ and H$_2$O$_2$ formation cause excessive accumulation of cellular iron (39,23). Most of the cellular iron is used to synthesize iron-sulfur proteins and cytochromes in mitochondria (40). Thus, inactivation of mitochondrial iron-sulfur proteins (e.g., aconitase and complex-I) is usually a harbinger for iron signaling. Similarly, MPP$^+$-mediated inactivation of mitochondrial iron sulfur proteins is likely responsible for stimulating the cellular iron-sensing mechanism by inducing the expression of TfR and associated iron uptake. Consistent with this mechanism, there was a decrease in the aconitase activity and an increase in TfR expression as early as 4-6 h following MPP$^+$ treatment in neuroblastoma cells (Fig. 2 A and B). However, we did not notice any significant increase in TfR mRNA levels under similar treatment conditions (Fig. 2C). This could be due to its relatively higher half-life (41). It is also possible that additional regulatory mechanisms are involved for TfR protein expression, in addition to IRP1 activation as described earlier.
(41). However, these events precede apoptosis as measured by caspase 3 activation (Fig. 2D). Agents that chelate intracellular iron, over-expression of GPx1 (which scavenges H$_2$O$_2$) and TfR antibody (that binds to the extracellular domain of TfR and inhibits iron entry into cells) significantly down-regulated MPP$^+$-induced α-syn at both transcriptional and translational levels and subsequent apoptotic signaling (Fig. 3). To confirm that MPP$^+$-induced H$_2$O$_2$ is responsible for α-syn up-regulation, we treated neuroblastoma cells with H$_2$O$_2$ and found the induction of both α-syn mRNA and protein levels prior to apoptosis (Fig. 4). A recent report showed that intracellular iron and TfR over-expression via an iron sensing mechanism involving IRP triggered H$_2$O$_2$-induced oxidative stress and apoptosis (42). Consistent with this mechanism, iron chelation by over-expression of the iron binding protein ferritin or administration of an iron chelator (clioquinol) to mice dramatically inhibited MPTP-induced oxidative damage in dopaminergic cells and behavioral deficits (43).

α-Synuclein is a protein containing 140 amino acids with 4 tyrosine residues. Under nitrative and oxidative stress, α-syn undergoes oxidation and oligomerization through di-tyrosyl formation (44). Collectively, these findings imply that MPP$^+$-induced α-syn aggregation in neuroblastoma cells is likely mediated by oxidants generated from iron and H$_2$O$_2$. At present, we do not know how MPP$^+$ induced oxidative stress and iron plays a role in up-regulation of α-syn mRNA, but it is possible that the 5’ and 3’ UTR of α-syn may be regulated by an iron-sensing mechanism similar to that proposed recently for the amyloid precursor protein (APP) (45).
Antisense α-syn abrogated MPP⁺-induced apoptosis but not oxidative stress —

Although there are conflicting data with regard to the potential toxicity of α-syn over-expression in non-dopaminergic neuronal cells, α-syn over-expression in dopaminergic neurons consistently leads to increased cytotoxicity (18). Moreover, α-syn null mice were found to be resistant to MPTP toxicity (46). As also shown in this study, α-syn over-expression alone leads to apoptosis in neuroblastoma cells: however, we did not detect a significant change in mitochondrial complex-1 and aconitase activities in α-syn over-expressing cells (Fig. 5C and D). The addition of MPP⁺ to α-syn over-expressing cells enhanced apoptosis as compared to MPP⁺ treatment alone (Fig 5 B). As MPP⁺ induced α-syn expression prior to the onset of apoptosis, we examined the effect of antisense α-syn treatment on MPP⁺-induced oxidative stress and apoptosis. Interestingly, although the antisense α-syn treatment significantly abrogated MPP⁺-induced apoptosis, oxidant generation continued unabated in these cells, as measured by the inactivation of aconitase and complex-1 activities and enhanced intracellular DCF fluorescence (Fig. 5). Thus, α-syn likely exerts its effects in neuroblastoma cells subsequent to oxidative stress and not vice versa.

Possible involvement of MAP kinase signaling and proteasome pathways in α-syn mediated apoptosis — Recent data show that monomeric and aggregated forms of α-syn inhibit the proteasomal function at nanomolar concentrations by interacting with the 26S and 20S proteasomes (31) and that the proteasomal inhibition further exacerbated MPP⁺-induced toxicity (47). Our present findings indicate that the trypsin-like activity of the proteasome was significantly inhibited by both MPP⁺ treatment and α-syn over-
expression. In α-syn under-expressing cells, these effects were abrogated (Fig. 6B), implicating that the proteasomal dysfunction could play a critical role in α-syn-mediated apoptosis. Earlier reports indicated that α-syn accelerates cell death by inhibiting MAP kinase activity (30). Our findings also imply that α-syn over-expression as well as MPP⁺ treatment induce apoptosis by inhibiting ERK/MAP kinase pathway, as cells pretreated with U0126, a specific inhibitor of ERK/MAP kinase, abrogated the protective effects induced by antisense α-syn transfection (Fig 6A). As proteasome inhibitors were also shown to block ERK signaling (48), it is not entirely clear whether these two mechanisms, as proposed in this study, are mutually exclusive or synergistically active.

In conclusion, as summarized in Scheme 1, the present findings demonstrate that MPP⁺ induces α-syn expression at both transcriptional and translational levels through intracellular H₂O₂ formation and intracellular iron sensing mechanism(s). Agents that scavenge H₂O₂ or prevent intracellular iron accumulation prevents MPP⁺-induced α-syn expression and apoptosis. α−Syn promotes apoptosis downstream to oxidative stress, possibly involving proteasomal dysfunction and / or MAP kinase inhibition.

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Figure legends

Figure 1. **MPP⁺ up-regulates α-syn expression**- (A) Neuroblastoma cells were incubated with 5 mM MPP⁺ for different time intervals. Following the termination of incubation, cells were collected by gentle scraping, washed thrice with DPBS and lysed in RIPA buffer. α-Syn levels were determined by Western blotting using monoclonal antibodies raised against α-syn followed by a secondary antibody coupled to HRP. Bands were visualized by the ECL detection method, (B) The densitometric analysis of gels shown in (A) using an Alpha Imager software, and (C) shows α-syn mRNA levels under the same experimental conditions as described in (A). After terminating the incubation, the media were aspirated and 1 ml of TRIzol reagent was added to cells and the lysate was collected. Total RNA was isolated from the cell lysate and RT-PCR was performed using the gene specific primers for α-syn as described in “Materials and Methods”. 18s rRNA was used as a loading control. Samples were resolved on a 1.5% agarose gel in TAE buffer, the images were captured using an Alpha Imager gel documentation system, and band intensities were calculated by the densitometric analysis using an Alpha Imager software. Data shown is the representative of three different experiments. Values indicated are the mean ± SD of three separate experiments. *p<0.05 compared to control.

Figure 2. **MPP⁺-induces aconitase inactivation, TfR expression, and caspase-3 activation.** (A) Neuroblastoma cells were treated with 5 mM MPP⁺ for different time intervals. The cells were collected by gentle scraping, washed thrice with DPBS and lysed. Aconitase activity in cell lysates was measured by monitoring the conversion of isocitrate to cis-aconitate at 240 nm as described in “Materials and Methods”, (B) Under
these conditions, the TfR protein expression was analyzed by Western blotting using the monoclonal antibodies raised against TfR followed by a secondary antibody coupled to HRP. Bands were visualized by an ECL detection method. The band intensity was calculated by the densitometric analysis using an Alpha Imager software, (C) The RT-PCR analysis of TfR mRNA was performed using the gene specific primers for TfR. 18s rRNA was used as a loading control. The samples were resolved on 1.5% agarose gel in TAE buffer and the images were captured using an Alpha Imager gel documentation system. Band intensity was calculated by densitometric analysis using Alpha Imager software. (D) The caspase 3 activity was determined in the supernatant of cell lysates isolated from experiment described in (A). Data shown are the mean ± SD of three separate experiments. *p<.01 compared to control.

**Figure 3. Treatment with iron chelator, antioxidant, and antioxidant enzyme suppresses MPP⁺-induced α-syn expression, oxidative stress, and apoptosis.** Neuroblastoma cells were incubated with 5 mM MPP⁺ in the presence or absence of TfR antibody, HBED, L-NAME, FeTBAP and GPx1 overexpression. After terminating the incubation, cells were collected by gentle scraping, washed thrice with DPBS and (A) aconitase activity was analyzed by monitoring the absorbance at 240 nm as described in Methods section, (B) Cells treated under the same incubation conditions as above were lysed in RIPA buffer and 50 µg of protein was resoled on 10% SDS-PAGE and Western blotting was performed to analyze α-syn levels as described in “Materials and Methods”, (C) Total band intensities of each lane in (B) were calculated by Alpha Imager gel documentation system, (D) RT-PCR analysis of α-syn levels from total RNA isolated using TRIzol reagent. as described in “Materials and Methods”. Following 35 cycles of
PCR, samples were resolved in 1.5% agarose gel in TAE buffer, images were captured and the intensity of bands was calculated by the densitometric analysis using an Alpha Imager software and (E) Neuroblastoma cells were treated with various agents as described in (A) and the caspase-3 activity was measured in cell lysates using an ApoAlert caspase 3 activity kit (Clontech) according to the manufacturer’s protocol. Data shown are the representative of three different experiments. Values indicated are the mean ± SD of three separate experiments. For A and E, *p<0.01 compared to control and #p<0.01 compared to MPP+-treated group. For B, C and D *p<0.05 compared to controls; #p<0.05 compared to MPP+ treated group.

Figure 4. Exogenously added H₂O₂ enhances α-syn expression and apoptosis.
Neuroblastoma cells were incubated with 150 µM H₂O₂ for different time intervals. Following the termination of incubation, cells were collected by gentle scraping, washed thrice with DPBS and lysed in RIPA buffer. (A) Western blotting was performed as described in “Materials and Methods”, employing monoclonal antibodies raised against α-syn followed by ECL detection method. (B) Band intensity of gels obtained under conditions described in (A) was calculated by densitometric analysis using an Alpha Imager gel documentation system. (C) RT-PCR was performed following isolation of total RNA by TRIzol reagent and first strand cDNA synthesis as described in “Materials and Methods”. Following PCR samples were resolved in 1.5% agarose gel in TAE buffer and bands were visualized under UV transilluminator and band intensity was calculated by densitometric analysis using Alpha Imager gel documentation system. (D) Caspase 3 activity in the supernatants of cell lysates was measured using ApoAlert
caspase 3 activity kit as mentioned in “Materials and Methods”. Data shown are the mean ± SD of three separate experiments. *p<0.01 compared to control

Figure 5. The effect of α-syn over-expression and depletion on MPP⁺-induced oxidative stress and apoptosis. (A) Western blot demonstrating the levels of α-syn levels in neuroblastoma cells following transfection of pcDNA 3 plasmid encoding the wild-type and antisense α-syn. The densitometric analysis of α-syn levels was calculated using an Alpha Imager gel documentation system. (B) Neuroblastoma cells transfected with α-syn and antisense α-syn plasmids were treated with 5 mM MPP⁺ for 12 h and caspase 3 activity was measured. (C) Incubation conditions are identical to those described in (A). Aconitase activity in cell lysates was measured by monitoring the conversion of isocitrate to cis-aconitate at 240 nm as described in “Materials and Methods”. (D) Incubation conditions are identical to those described in (A) and the complex-1 activity in cell lysates was measured as described in “Materials and Methods” by monitoring the disappearance of NADH in the presence of ubiquinone-1 at 340 nm. (E) Following the termination of incubations described under (B), cells were incubated with 10 μM carboxy-2HDCFDA for 30 min. The medium was subsequently aspirated and cells were washed twice with DPBS and fluorescence images were captured in a Nikon fluorescence microscope equipped with FITC filter settings. (F) The fluorescence intensity of carboxy-DCF was calculated using a Metamorph software. Values indicated are the mean ± SD of three separate fields of view. *p<0.01 compared to control, #p<0.01 compared to MPP⁺ treated group.

Figure 6. The effect of α-syn on MAPK and proteasomal activities. Neuroblastoma cells were incubated with 5 mM MPP⁺ for 12 h in the presence and absence of α-syn
over-expression and antisense α-syn treatment with or without the MAP kinase inhibitor U0126 (25 μm). Following the termination of experiment, cells were washed twice with DPBS and (A) caspase 3 activity was measured as described in the “Methods Section” using the ApoAlert caspase 3 activity kit (Clontech). Data shown are the mean ± SD of three separate experiments, and (B) The trypsin-like activity of 26s proteasome function was measured as described in the “Methods Section”. Briefly, after terminating the incubation cells were washed with buffer I (50 mM Tris, pH 7.4, 2 mM DTT, 5 mM MgCl₂, 2 mM ATP) and homogenized with buffer I containing 250 mM sucrose. Twenty μg of 10,000 X g supernatant were diluted with buffer I to a final volume of 900 μl. The fluorogenic proteasome substrate Z-Leu-Leu-Lys-AMC (trypsin-like) was added in a final concentration of 80 μM. The proteolytic activity was measured by monitoring the release of the fluorescent group 7-amido-4-methylcoumarin (excitation and emission at 380/460 nm). *p<0.01 compared to control, #p<0.01 compared to MPP⁺ treated group and †p<0.01 compared to MPP⁺ plus AS-α-syn treated group

Scheme 1. Schematic representation of the interrelationships among MPP⁺-induced oxidative stress, IRP-regulated TfR-dependent transferrin iron, hydrogen peroxide generated from the inhibition of complex-1, α-syn expression/aggregation, proteasomal (dys)function, and apoptotic signaling. As shown in this scheme, cell-permeable iron-chelators, antioxidant (enzyme), and ROS scavengers mitigate MPP⁺-induced apoptosis in neuroblastoma cells.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Scheme 1

MPP⁺

↓ Complex 1

↓ Superoxide

↑ H₂O₂

FeTBAP

↓ Aconitase

↑ H₂O₂

FeTBAP

↓ Aconitase

↑ H₂O₂

FeTBAP

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IRP/IRE binding was measured by electrophoretic mobility shift assay. $^{32}$P-Labeled IRE mRNA for the RNA band shift assay was prepared using as a template a 1000-base pair rat L ferritin pseudogene that contains the conserved IRE sequence. The plasmid ($p66-L$ gene) containing this insert (which was generously provided by Dr. Elizabeth Leibold) was linearized with $SmaI$ (Invitrogen) and used for *in vitro* transcription of IRE mRNA. Transcription was carried out with Sp6 RNA polymerase using a Riboprobe transcription system from Promega. To determine whether the increase in IRP-1 activity with MPP$^+$ treatment was due to an increase in total IRP-1, lysates were treated with 1% 2-mercaptoethanol, which activates IRP-1 to the high affinity RNA-binding form.
Figure 2: Half-life of TfR mRNA in the presence or absence of MPP⁺ treatment

Cells were incubated with 10µg/ml of actinomycinD for indicated time intervals in the presence or absence of 5mM MPP⁺ treatment for 6 hours. After the termination of incubation total cellular RNA was isolated by TRIzol reagent and RT-PCR was performed. Densitometric analysis was performed using Alpha-Innotech gel documentation system.
a-Synuclein up-regulation and aggregation during MPP+-induced apoptosis in neuroblastoma cells: Intermediacy of transferrin receptor iron and hydrogen peroxide
Shasi V. Kalivendi, Sonya Cunningham, Srigiridhar Kotamraju, Joy Joseph, Cecilia J. Hillard and B. Kalyanaraman

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