Activation of apoptosis signal regulating kinase 1 (ASK1) and translocation of death-associated protein, Daxx, in substantia nigra pars compacta in a mouse model of Parkinson's disease: protection by α-lipoic acid

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ABSTRACT **Parkinson's disease (PD), a neurodegenerative disorder, causes severe motor impairment due to loss of dopaminergic neurons in substantia nigra pars compacta (SNpc). MPTP, a neurotoxin that causes dopaminergic cell loss in mice, was used in an animal model to study the pathogenic mechanisms leading to neurodegeneration. We observed the activation of apoptosis signal regulating kinase (ASK1, MAPKKK) and phosphorylation of its downstream targets MKK4 and JNK, 12 h after administration of a single dose of MPTP. Further, Daxx, the death-associated protein, translocated to the cytosol selectively in SNpc neurons seemingly due to MPTP mediated down-regulation of DJ-1, the redox-sensitive protein that binds Daxx in the nucleus. Coadministration of α-lipoic acid (ALA), a thiol antioxidant, abolished the activation of ASK1 and phosphorylation of downstream kinases, MKK4, and JNK and prevented the down-regulation of DJ-1 and translocation of Daxx to the cytosol seen after MPTP. ALA also attenuated dopaminergic cell loss in SNpc seen after subchronic MPTP treatment. Our studies demonstrate for the first time that MPTP triggers death signaling pathway by activating ASK1 and translocating Daxx, *in vivo*, in dopaminergic neurons in SNpc of mice and thiol antioxidants, such as ALA terminate this cascade and afford neuroprotection.—Karunakaran, S., Diwakar, L., Saeed, U., Agarwal, V., Ramakrishnan, S., Iyengar, S., Ravindranath, V. Activation of apoptosis signal regulating kinase 1 (ASK1) and translocation of death associated protein, Daxx in substantia nigra pars compacta in a mouse model of Parkinson's disease: Protection by α-lipoic acid. *FASEB J.* 21, 2226–2236 (2007)**

Key Words: MAPK signaling · MPTP · DJ-1 · oxidative stress · cell death

Neurodegenerative disorders are a heterogeneous group of disorders with onset typically in the fifth to seventh decade of life. They are progressive in nature and affect selective regions of the CNS. A common pathological hallmark of neurodegenerative disorders such as amyotrophic lateral sclerosis, Alzheimer's disease, Huntington's disease, and Parkinson's disease (PD) is the loss of subsets of neurons within specific regions of the CNS. Severe motor impairment is seen in PD, the second most common neurodegenerative disorder after Alzheimer's disease. The clinical symptoms include bradykinesia, rigidity, and resting tremor. PD is associated with a profound and selective loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) of the midbrain and the dopaminergic terminals in the striatum. At the time of clinical presentation, ~50–70% of dopaminergic neurons in the nigrostriatal system are lost (1). Oxidative stress and mitochondrial dysfunction caused by loss of complex I activity are identified as some of the primary events leading to neurodegeneration in PD.

Exposure to MPTP (1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine), a model neurotoxin leads to loss of dopaminergic neurons in SNpc in mice in a manner similar to that seen in Parkinson's disease. MPTP is, therefore, used as a model neurotoxin to study the molecular events that cause degeneration of dopaminergic neurons in Parkinson's disease (2). In the brain, MPTP is oxidized by monoamine oxidase B to MPP^+, the ultimate toxic metabolite. MPP^+ is a high-affinity substrate for the dopamine transporter (3) and is taken up selectively into dopaminergic neurons. MPP^+ generates oxidative stress and impairs oxidative phosphorylation by inhibiting complex I of the mitochondrial electron transport chain (4), leading to rapid decrease in ATP content in the striatum and ventral midbrain,

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the brain regions most sensitive to MPTP toxicity (5). Significant ATP depletion can result from as little as 25% inhibition of complex I (6), indicating a causal relationship between complex I activity and ATP production in mitochondria.

Earlier studies from our laboratory have demonstrated that administration of MPTP to male mice results in loss of the antioxidant thiol glutathione, measured as sum of oxidized (GSSG) and reduced GSH, which precedes the inhibition of mitochondrial complex I activity in midbrain (7). Complex I inhibition caused by MPTP can be reversed by dithiothreitol, providing further evidence that protein thiol oxidation is a primary cause for complex I inhibition (8). More recently we have also demonstrated that glutaredoxin (also known as thioltransferase; Grx1), a thiol-disulfide oxidoreductase that efficiently and specifically reduces glutathione-containing mixed disulfides (9), is required for maintenance of complex I function in normal conditions. Its upregulation is critical for the recovery of complex I function following MPTP administration, indicating that complex I dysfunction following MPTP presumably occurs through glutathionylation of its critical thiol groups.

Generally, oxidative stress in brain results in extensive oxidative modification of protein thiols at available cysteine residues in proteins (10). Although such oxidative modification of thiols, including glutathionylation of proteins, is seen in certain cells of non-neural origin (11), it is more pronounced in brain, presumably because extrusion of GSSG, as routinely observed in other tissues such as lung and liver is not always seen in brain (12). Oxidative modification of protein thiol groups, through formation of mixed disulfides, can impair the functional characteristics of critical proteins. For example, formation of PrSSG can potentially disrupt mitochondrial function by cross-linking sulfhydryl groups, resulting in the opening of the mitochondrial permeability transition pore (13). More importantly, redox status of critical thiol groups in signaling molecules such as kinases and phosphatases often determine the initiation and progression of signaling cascades, which decide cell fate in terms of activating and propagating death *vs.* survival pathways (14). Thus, ASK1, a mitogen-activated protein kinase kinase kinase (MAPKKK) exists in cells in association with thioredoxin (Trx), which prevents it from autophosphorylation. Oxidative modification of thioredoxin results in its dissociation from ASK1 leading to activation of ASK1 by autophosphorylation (15, 16). ASK1 then initiates the MAP kinase cascade, leading to the phosphorylation of c-Jun N-terminal kinase (JNK). Most of the existing knowledge on redox signaling has emerged from the cultured cell systems following overexpression of recombinant proteins. However, their physiological relevance, *in vivo*, remains to be established.

In the current study, we examined the status of the MAP kinase-signaling cascade in striatum and ventral midbrain following administration of MPTP to mice. In addition, we coadministered the thiol antioxidant, α-lipoic acid (ALA) with MPTP and examined its effect on the MAPK signaling and subsequent neuroprotection.

MATERIALS AND METHODS

Materials

The antibodies to Daxx (M-112, polyclonal), ASK-1 (H-300, polyclonal), pJNK (G-7, monoclonal), and DJ-1 (C-16, polyclonal) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibody to β-tubulin was obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibody to lamin A/C and phospho-SEK1/MKK4 (Thr261) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Vectastain-ABC Elite kit was purchased from Vector labs (Burlingame, CA, USA). All other chemicals and reagents were of analytical grade and were obtained from Sigma Aldrich or Qualigens (India).

Animals

All animal experiments were carried out as per the institutional guidelines for the use and care of animals. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques if available. Male C57BL6J and Swiss albino mice, (2 to 3 mo, 25–30 g) were obtained from National Brain Research Centre (NBRC) and Central Animal Research Facility of National Institute of Mental Health and Neurosciences (NIMHANS). Animals were given MPTP (30 mg/kg body weight) subcutaneously. Animals had access to pelleted diet and water *ad libitum*. Animals were also administered with sodium salt of α-lipoic acid (ALA; 22 mg/kg body weight, s.c.) twice daily. Control animals received vehicle alone. At the end of the experimental period, mice were anesthetized with ether and perfused transcardially with ice-cold normal saline before decapitation. Ventral midbrain was dissected as described (**Fig. 1**). Ventral midbrain and striatum were frozen in liquid nitrogen for immunoblotting, assay of GSH and complex I activity. Animals were perfused transcardially with buffered paraformaldehyde (4% w/v), and the brain was dissected out and processed for immunohistochemistry. All experiments were carried out on C57 black mice unless indicated otherwise. Brain regions from 2–3 animals were pooled for each sample.

Processing of tissue

Tissue was homogenized in 0.25 M sucrose and centrifuged at 1000 g for 10 min to obtain postnuclear supernatant. The postnuclear supernatant was used for immunoblotting. Postnuclear supernatant was centrifuged again at 14,000 g for 30 min to obtain the mitochondrial pellet. The pellet was suspended in sucrose (0.25 M) and freeze-thawed for assay of complex I. The postnuclear supernatant was also centrifuged at 100,000 g for 30 min to obtain cytosol, which was used for estimation of cystathionine-γ-lyase activity. Protein concentration was estimated by a dye-binding method (17). For the assay of GSH, tissues were frozen immediately in liquid nitrogen and homogenized in 9 volumes of 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA. An aliquot of homogenate was added to an equal volume of 5-sulfosalicylic acid (1%, w/v), mixed and centrifuged at 10,000 *g* for 10 min, and the supernatant was used for estimation of total GSH and the pellet for PrSSG using HPLC.

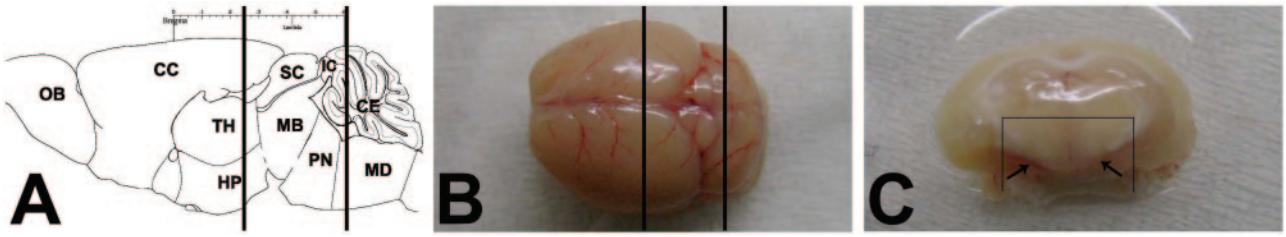

Figure 1. Dissection of the ventral midbrain region from mice. Bregma coordinates and anatomic boundaries were established for dissection of ventral midbrain based on the mouse brain atlas (42). Briefly, the skull plates were peeled away to visually locate the bregma. The brain was removed from the skull and placed dorsal side up. Using a scalpel blade, a coronal cut was made adjacent to the inferior colliculi approximately at bregma –6.36 mm. A second cut was made approximately at bregma –2.54 mm, depicted by solid lines in (*A*) and (*B*). The slice was placed rostral side up as indicated in (*C*). The ventral midbrain was dissected out as indicated by solid lines in (*C*) ensuring that there was no contamination of the hippocampus, cortex or cerebellum. The arrows in (*C*) indicate substantia nigra pars compacta.

Estimation of total GSH and PrSSG

GSH and PrSSG were estimated by HPLC following derivatization with fluorodinitrobenzene (FDNB) (18) using γ-glutamylglutamate (γ-Gluglu) as internal standard. For assay of GSH, the acid-soluble supernatant was treated with FDNB and used directly for HPLC analysis. For assay of PrSSG, the acid-insoluble pellet was washed with ethanol and treated with MOPS-dithiothreitol buffer to reduce PrSSG to protein thiol (18). The GSH thus released was derivatized and subjected to HPLC analysis.

Estimation of MPP^+

Ventral midbrain was dissected out from animals treated with MPTP alone or ALA and MPTP for 1 and 12 h as described above. The brain tissue was homogenized in potassium phosphate buffer (50 mM, pH 7.4), mixed with methanol (1:1 v/v), vortexed, centrifuged at 14000 g, and filtered through 0.45 μm membrane. The filtrate (100 μl) was injected into HPLC column (SUPELCOSIL, LC-CN). The mobile phase consisted of 0.1 M sodium acetate buffer (pH 7.2) containing 10% acetonitrile (v/v), 0.1% triethylamine (v/v), and tetraethyl ammonium bromide (0.01%, w/v). MPP^+ was detected using UV detector set at 292 nm (19) and quantitated from the standard curve run using known concentrations of MPP^+.

Assay of NADH: ubiquinone oxido-reductase (complex I) activity

Complex I was assayed in mitochondrial preparations as rotenone-sensitive NADH-ubiquinone oxido-reductase (20). The assay was performed in 35 mM potassium phosphate buffer (pH 7.4) containing sodium cyanide (2.65 mM), magnesium chloride (5 mM), EDTA (1 mM), bovine serum albumin (1 mg/ml), and antimycin (2 μg/ml). Brain mitochondria (70–80 μg) and ubiquinone 1 (0.05 mM) were added to the assay buffer to a final volume of 0.48 ml. After preincubation of the reaction mixture at room temperature for 2 min, the reaction was initiated by addition of 0.02 ml of 5 mM NADH solution. Test and blank reactions were run separately. The rate of decrease of absorbance at 340 nm was monitored over 3 min. The assay was also performed in the presence of rotenone to determine rotenone-sensitive enzyme activity. Less than 2% of the total activity was rotenone insensitive. The enzyme activity is expressed as nanomoles of NADH oxidized/min/mg protein.

Assay of cystathionine-γ-lyase

Cystathionine-γ-lyase activity was measured in cytosol prepared from mouse brain or liver (21). The reaction mixture (total volume 0.5 ml) containing cytosolic protein from brain (2 mg), 100 μl Tris-HCl buffer (5 mM), and 10 μl of pyridoxal 5′-phosphate (1 mM) was considered as blank. The test sample contained all the above and 10 μl of L-cystathionine (2 mM) as substrate. After 2 h of incubation at 37°C, the tubes were placed in an ice-water bath at 4°C to terminate the reaction. Dithiothreitol (5 mM, 10 μl) was added to each tube to reduce cystine to cysteine. Ninhydrin reagent was added to the tubes and heated in boiling water bath for 10 min. Tubes were cooled under tap water, and the absorbance of the pink colored complex was measured at 560 nm by spectrophotometric method (22) using cysteine as standard.

Immunohistochemistry and stereology

Male C57BL6J mice were administered MPTP (30 mg/kg body weight/day, s.c.) once daily for 1 or 8 d. Control animals received saline. Animals were anesthetized with ether 24 h after the last injection and perfused transcardially with phosphate-buffered saline (PBS) followed by paraformaldehyde (4%, w/v) in PBS and postfixed in paraformaldehyde. Cryostat-cut coronal sections (30 μm thick) were prepared throughout the entire midbrain from a random start position, and every fifth section was processed for tyrosine hydroxylase immunohistochemistry. Sections were visualized after incubation with secondary antibody conjugated to FITC under fluorescent microscope. Persons blind to the treatment counted the number of tyrosine hydroxylase positive neurons in the substantia nigra pars compacta (SNpc). Sections passing through rostral, middle and caudal regions of the SN, were examined. The pars compacta region was delineated for stereological counting. This delineation excluded pars reticulata (SNR), ventral tegmental area and the retrorubral area.

For immunostaining of Daxx, sections were exposed to hydrogen peroxide (3% v/v) to block the endogenous peroxidase reaction and then processed using antiserum to Daxx. Finally, sections were washed, treated with biotinylated anti-rabbit IgG (diluted 1: 500 in PBS) for 1 h, and incubated with VECTASTAIN-Elite ABC reagent. The color was developed using diaminobenzidine and hydrogen peroxide. In a separate set of experiments the immunostaining was visualized using FITC labeled secondary antibody and the sections were counterstained with DAPI.

Studies with SH-SY5Y cells

SH-SY5Y cells were cultured in MEM (Gibco BRL) supplemented with 10% FBS. Cells were seeded in chamber slide at 70% confluency prior to treatment with MPP^+ (3 mM) for 12 h. In experiments showing reversal of Daxx translocation, cells were treated with N-acetyl cysteine (100 μM) and ALA (100 μM), 60 min prior to addition of MPP^+. Cells were then washed with PBS and fixed with 4% paraformaldehyde at room temperature for 30 min. Immunocytochemistry for Daxx was performed after washing the cells with PBS and permeabilization with digitonin (0.01%; w/v) followed by overnight incubation with antiserum to Daxx. After washing, cells were incubated with secondary antibody conjugated to Alexa flour 594, washed, mounted in medium containing DAPI, and visualized under a fluorescence microscope.

Immunoblotting

The postnuclear supernatant prepared from ventral midbrain or striatum (20 μg protein) of vehicle and treated mice was resolved on 10% sodium dodecyl sulfate polyacrylamide gel (23). Proteins were transferred to nitrocellulose membranes (24), incubated with primary antibody (1:1000) followed by secondary antibody (1:2000) labeled with alkaline phosphatase. Immunostained bands were detected using nitroblue tetrazolium and 5-bromo 4-chloro 3-indolyl phosphate as chromogens.

Statistical analysis

Statistical analysis of the data was performed using analysis of variance followed by Student-Newman-keuls or Duncan's test or *post hoc* Students' *t* test, as appropriate. Values of $P < 0.05$ were taken as being statistically significant.

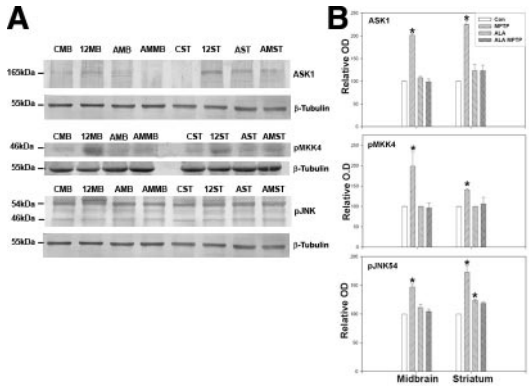

Figure 2. Coadministration of ALA attenuates MPTP induced activation of ASK1, pMKK4 and pJNK. Animals were treated with a single dose of vehicle or MPTP and sacrificed 12 h later. Some animals also received a bidaily dose of ALA. *A)* Representative blots from ventral midbrain (MB) and striatum (ST) of animals treated with saline (lane 1), MPTP (lane 2), ALA (lane 3), and ALA + MPTP (lane 4), depicting the protein levels of ASK1, phospho-MKK4 (p-MKK4), and phospho-JNK (p-JNK) as determined by immunoblot are presented. β-tubulin levels were measured as loading control. *B)* Densitometric analysis of the immunoblots representing the relative intensity of the immunoreactive bands are shown. Values are mean ± SD (n=3 animals). Asterisks indicate values significantly different from corresponding control (P<0.05). The lanes are labeled as CMB—Control midbrain; 12MB—12 h MPTP treated midbrain; AMB—ALA treated midbrain; AMMB—ALA + MPTP treated midbrain; CST—control striatum; 12ST—12 h MPTP treated striatum; AMB—ALA treated striatum; AMMB—ALA + MPTP treated striatum.

RESULTS

ALA reverses MPTP mediated activation of ASK1, MKK4, and JNK

We examined the phosphorylation states of MAP kinases following MPTP administration and further studied the effect of coadministration of α-lipoic acid. Both in striatum and ventral midbrain, we observed the activation of ASK1 and phosphorylation of its downstream targets, MKK4 and JNK, 12 h after MPTP administration. Animals treated with both ALA and MPTP were similar to controls, and the phosphorylation states of MKK4 and JNK were unaffected (**Fig. 2*A*, *B***) indicating clearly that α-lipoic acid prevents the activation of the proapoptotic signaling pathway initiated by ASK1. This is in consonance with our earlier results that indicate that ALA helps maintain thiol homeostasis in brain (25).

GSH and PrSSG levels in striatum and midbrain after MPTP administration

GSH and PrSSG levels were estimated using HPLC in striatum and midbrain regions of Swiss albino mice treated with MPTP for 4 h (**Fig. 3*A***). The chromatogram (Fig. 3*A*) from the striatum of vehicle and MPTP-treated mice shows the internal standard γ-glutamylglutamate (γ-Gluglu) followed by GSH or PrSSG assayed as GSH, which were derivatized and separated as dinitrophenyl derivatives on octadecyl amino propyl column by reverse-phase HPLC. MPTP administration leads to loss of GSH and increase in GSH levels recovered as PrSSG.

Decrease in GSH levels (32% in striatum and 21% in midbrain) and increase in PrSSG (123% in striatum and 118% in midbrain) were seen (Fig. 3*B*). In striatum and midbrain the amount of GSH lost was essentially recovered as PrSSG, indicating that MPTP exposure results in significant perturbation of the GSH-protein thiol homeostasis and extensive glutathionylation of protein thiols.

Effect of L-propargylglycine on γ-cystathionase activity, GSH levels, and complex I activity in mouse brain

Propargyl glycine (PPG) inhibits cystathionine γ-lyase (the rate-limiting enzyme in the conversion of methionine to cysteine), resulting in impaired synthesis of cysteine and thereby decreasing GSH synthesis (25, 26). We examined the effect of PPG on GSH levels and complex I activity in brain of Swiss albino mice. γ-Cystathionase activity in brain was inhibited significantly (68%) 18 h following a single dose of PPG (100 mg/kg

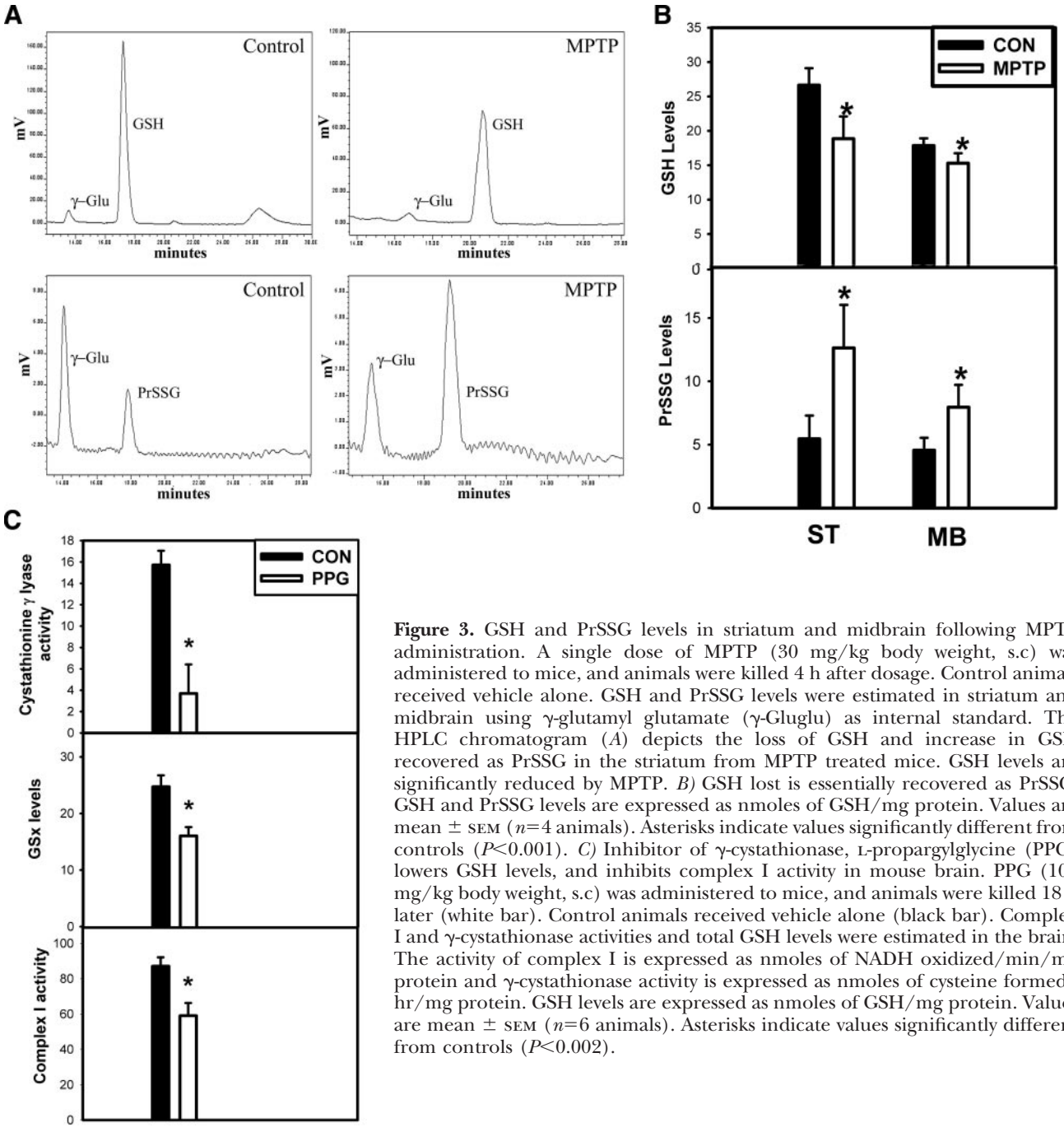

Figure 3. GSH and PrSSG levels in striatum and midbrain following MPTP administration. A single dose of MPTP (30 mg/kg body weight, s.c) was administered to mice, and animals were killed 4 h after dosage. Control animals received vehicle alone. GSH and PrSSG levels were estimated in striatum and midbrain using γ-glutamyl glutamate (γ-Gluglu) as internal standard. The HPLC chromatogram (*A*) depicts the loss of GSH and increase in GSH recovered as PrSSG in the striatum from MPTP treated mice. GSH levels are significantly reduced by MPTP. *B)* GSH lost is essentially recovered as PrSSG. GSH and PrSSG levels are expressed as nmoles of GSH/mg protein. Values are mean ± SEM (n=4 animals). Asterisks indicate values significantly different from controls (P<0.001). *C)* Inhibitor of γ-cystathionase, L-propargylglycine (PPG) lowers GSH levels, and inhibits complex I activity in mouse brain. PPG (100 mg/kg body weight, s.c) was administered to mice, and animals were killed 18 h later (white bar). Control animals received vehicle alone (black bar). Complex I and γ-cystathionase activities and total GSH levels were estimated in the brain. The activity of complex I is expressed as nmoles of NADH oxidized/min/mg protein and γ-cystathionase activity is expressed as nmoles of cysteine formed/hr/mg protein. GSH levels are expressed as nmoles of GSH/mg protein. Values are mean ± SEM (n=6 animals). Asterisks indicate values significantly different from controls (P<0.002).

body weight, s.c.). Concomitantly, total GSH (represented as GSx) were reduced by 33% and this also led to 28% loss in complex I activity (Fig. 3*C*). Thus, reduction in GSH levels, as seen after PPG can, *per se*, result in complex I inhibition.

Translocation of Daxx from nucleus to cytoplasm by MPP^+ in neuroblastoma cells and by MPTP, *in vivo*, in mice

Daxx, the death-associated protein is a transcriptional repressor that is normally present in the nucleus. Daxx is known to translocate to cytoplasm, associate with ASK1, and participate in the apoptotic process (27). We treated human neuroblastoma cells with MPP^+ and examined the subcellular localization of Daxx. Treatment with MPP^+ for 12 h resulted in the translocation of Daxx from nucleus to cytoplasm. However, when cells were treated with N-acetyl cysteine or ALA for 1 h prior to MPP^+, Daxx translocation could be prevented (**Fig. 4**). We observed a similar translocation of Daxx from the nucleus to the cytoplasm following MPTP administration, *in vivo* in mice. Daxx translocates from the nucleus to the cytosol in neurons in substantia nigra pars compacta (**Fig. 5**; top panel *B* and *C*) but not in the ventral tegmental area or substantia nigra pars reticulata (Fig. 5; middle panel). Daxx was present predominantly in the cytosol 24 h after administration of a

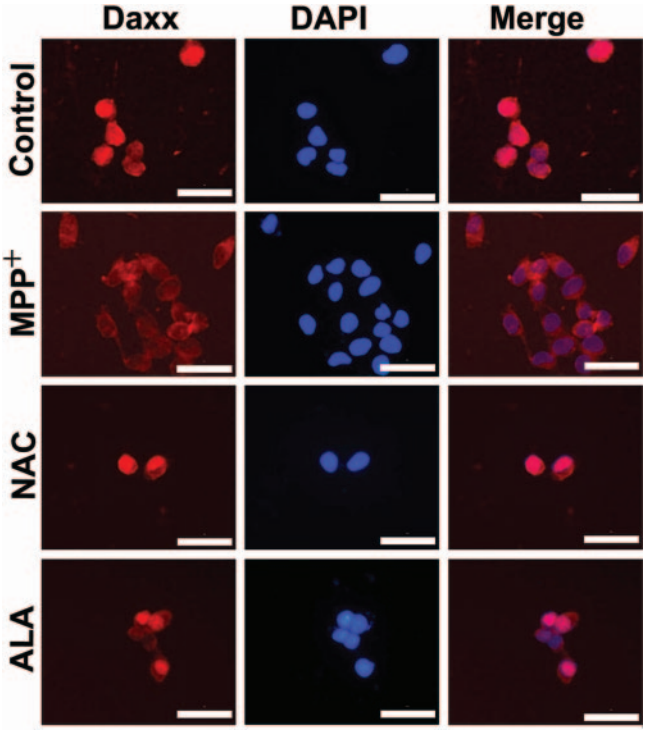

Figure 4. Daxx translocates from nucleus to the cytosol following exposure to MPP^+ in SH-SY5Y human neuroblastoma cells.Cells were exposed for 12 h to MPP^+ alone (3 mM) or in combination with N-acetyl cysteine (NAC; 100 μM) or ALA (ALA; 100 μM) and then immunostained for Daxx. Cells were counterstained with DAPI prior to mounting. On exposure to MPP^+ Daxx translocated to cytosol, which was prevented by prior treatment with NAC or ALA. Scale bar = 120 μm.

single dose of MPTP (Fig. 5; middle panel) and also in the surviving neurons after subchronic treatment of MPTP for 8 d (Fig. 5; middle panel). ALA coadministration could abolish these effects, and Daxx was essentially present in the nucleus (Fig. 5; middle panel). These observations were validated by visualizing Daxx immunostaining with FITC and costaining the sections with DAPI (Fig. 5; bottom panel), wherein the translocation of Daxx to the cytosol following MPTP administration is clearly discernable.

DJ-1 was down-regulated in a region specific manner following MPTP treatment

DJ-1, an antioxidant responsive protein interacts with Daxx in the nucleus and helps the sequestration of Daxx within the nucleus, preventing its translocation to the cytoplasm (28). We observed selective down-regulation of DJ-1 in the postnuclear supernatant prepared from ventral midbrain but not striatum following MPTP exposure, which was prevented by α-lipoic acid treatment (**Fig. 6*A***). Importantly, DJ-1 levels were also significantly lower in the nuclear extracts prepared

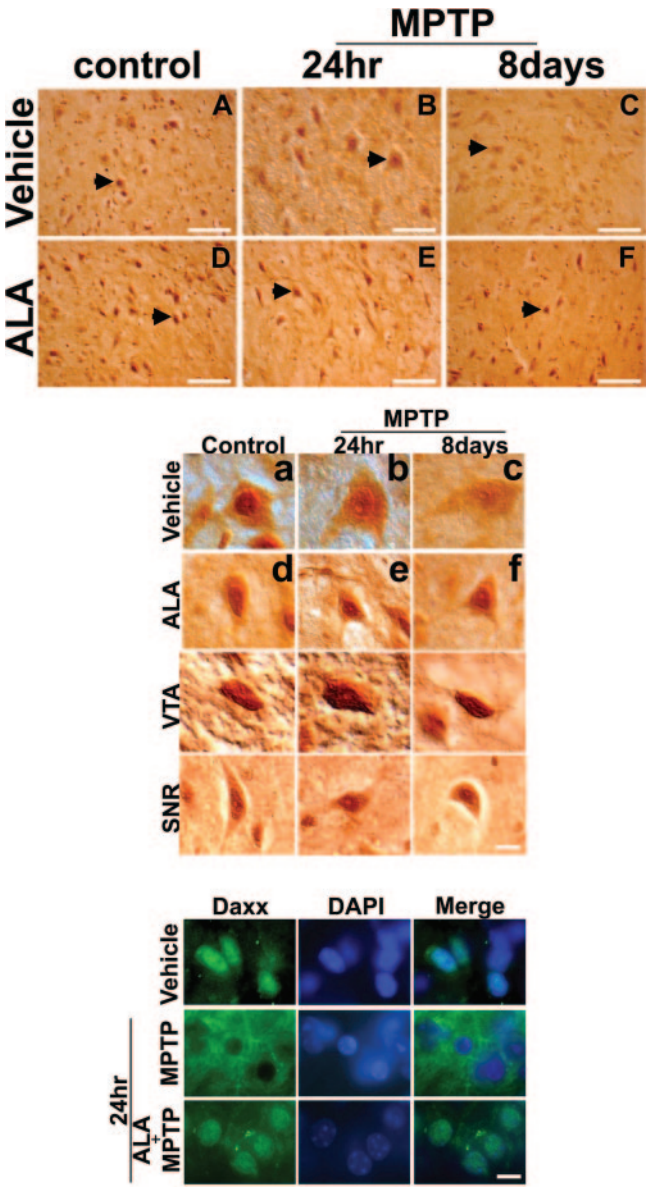

Figure 5. Translocation of Daxx to the cytosol in dopaminergic neurons of SNpc following administration of MPTP to mice. Animals were treated with a single dose of MPTP and sacrificed 24 h later. Animals were also treated with a daily dose of MPTP for 8 d and sacrificed on the 9th day. Two subsets of animals were coadministered α-lipoic acid twice daily. Immunohistochemical localization of Daxx revealed the translocation of Daxx to the cytosol in SNpc neurons after MPTP exposure. Daxx is present in the nucleus in control animals (*A*), while it is present in the cytoplasm following a single dose of MPTP (*B*), and in the surviving neurons after subchronic exposure to MPTP for 8 d (*C*). *D–F)* depict the subcellular localization of Daxx in corresponding group of animals coadministered ALA, wherein, Daxx is localized essentially in the nucleus. Scale bar = 25 μm. *a–f)* Of the lower panel show corresponding magnified images of the neurons indicated by arrowheads in the upper panel. The panels indicated by VTA and SNR depict the presence of Daxx in the nucleus in the ventral tegmental area (VTA) and substantia nigra pars reticulata (SNR) neurons following acute and subchronic MPTP exposure. Scale Bar = 10 μm. Immunofluorescent images for Daxx (green) also show the similar pattern of translocation of Daxx to the cytosol following a single dose of MPTP, which is prevented by ALA. Cells were counterstained with DAPI prior to mounting. Scale Bar = 10 μm.

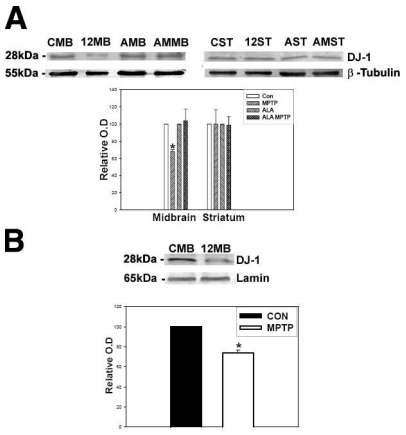

Figure 6. DJ-1 is down-regulated in ventral midbrain following MPTP treatment. Animals were treated with a single dose of vehicle or MPTP and sacrificed 12 h later. Some animals also received ALA twice daily. *A)* Representative immunoblot of extranuclear DJ-1 from ventral midbrain (MB) and striatum (ST) of animals treated with saline (lane 1), MPTP (lane 2), ALA (lane 3), and ALA + MPTP (lane 4) is depicted. β-Tubulin levels were measured as loading control. Densitometric analysis of the immunoblots representing the relative intensity of the immunoreactive bands DJ-1 bands shows the down-regulation of DJ-1 by MPTP which is prevented by ALA. *B)* DJ-1 levels in the nucleus are down-regulated 12 h after a single dose of MPTP. Representative immunoblot and densitometric analysis of the immunoblots representing the relative intensity of the immunoreactive bands of nuclear DJ-1 is depicted. Lamin was used as loading control. Values are mean ± SD (n=3 animals). *Asterisks* indicate values significantly different from corresponding control ($P<0.05$). The lanes are depicted as CMB—control midbrain; 12MB—12 h MPTP treated midbrain; AMB—ALA treated midbrain; AMMB—ALA + MPTP treated midbrain; CST—control striatum; 12ST—12 h MPTP treated striatum; AMB—ALA treated striatum; AMMB—ALA + MPTP treated striatum.

from ventral midbrain of MPTP treated animals (Fig. 6*B*).

ALA abolishes MPTP mediated complex I inhibition in male mice and attenuates dopaminergic cell loss in SNpc

We then examined the effect of coadministration of ALA on the number of surviving dopaminergic neurons in SNpc (**Fig. 7*A–D***) and mitochondrial complex I activity (Fig. 7*F*) following subchronic MPTP treatment for 8 d. Coadministration of ALA attenuated MPTP-mediated dopaminergic cell loss in SNpc measured by stereological analysis of dopaminergic neurons identified as tyrosine hydroxylase positive cells (Fig. 7*E*). In animals treated with MPTP alone, 24% of cell loss was seen after 8 d of MPTP (Fig. 7*B*), while in animals coadministered ALA, the cell loss was only 11%, indicating the significant protection offered by ALA (Fig. 7*E*). Coadministration of ALA with MPTP also abolished complex I inhibition in striatum and ventral midbrain, which is a hallmark of MPTP toxicity (Fig. 7*F*).

MPP^+ levels in ventral midbrain following MPTP administration

MPP^+ levels were measured in ventral midbrain at 1 and 12 h following a single dose of MPTP with and without ALA coadministration (**Fig. 8**). Significant amounts of MPP^+ were present in ventral midbrain 1 h after MPTP exposure (Fig. 8). Further, coadministration of ALA had no effect on MPP^+ levels in ventral midbrain, indicating that ALA did not alter the biodistribution of MPP^+. MPP^+ was not detectable 12 h after MPTP administration, indicating its rapid clearance from the ventral midbrain.

DISCUSSION

ASK1, a mitogen activated protein (MAP) kinase kinase kinase, is 160 kDA protein that functions as a serine threonine kinase. ASK1 is sensitive to reactive oxygen species and activates p38 and JNK by directly phosphorylating mitogen activated kinase kinases, MKK3/6 and MKK4/7 (15). ASK1 has two regulatory domains at the N- and C-terminal, while the kinase domain is present in the middle of the molecule. Thioredoxin (Trx) binds to the N-terminal domain and prevents the oligomerization and autophosphorylation of ASK1, which initiates the proapoptotic cascade. Overexpression of ASK1 (both wild-type and the constitutively active N-terminal truncated form) enhances ROS-mediated death in cultured cells, including that induced by 6-hydroxy dopamine (29), while apoptosis is suppressed in ASK1 knockout cells.

Exposure of mice to MPTP activated ASK1 and led to the phosphorylation of the downstream kinases namely, SEK1/MKK4 and JNK (Fig. 2). Phosphorylation of JNK has been identified as a key mediator of cell death in animal models of Parkinson's disease and pretreatment with JNK inhibitors attenuates dopaminergic cell loss (30, 31). However, there is no information on the upstream events leading to JNK activation. We show here that activation of ASK1 may be the trigger for JNK activation. Since oxidative modification of Trx dissociates it from ASK1, resulting in the activation of ASK1 through oligomerization and autophosphorylation (32, 33), we examined if the thiol antioxidant ALA could prevent ASK1 activation by presumably maintaining the redox status of Trx. ALA abolishes the activation of ASK1 mediated by MPTP, indicating that oxidative stress is presumably the trigger for ASK1 activation, resulting in phosphorylation of MKK4, which in turn phosphorylates JNK (Fig. 2).

Daxx, the death-associated protein and transcriptional repressor, translocates from the nucleus to the cytosol and associates with ASK1 propagating the death-signaling cascade (34). Daxx is presumably phosphorylated through ASK1-SEK1-JNK1 (35), which results in its translocation to the cytosol and association with ASK1. We examined the cellular status of Daxx, *in vitro*, using SH-SY5Y, the human neuroblastoma cell line.

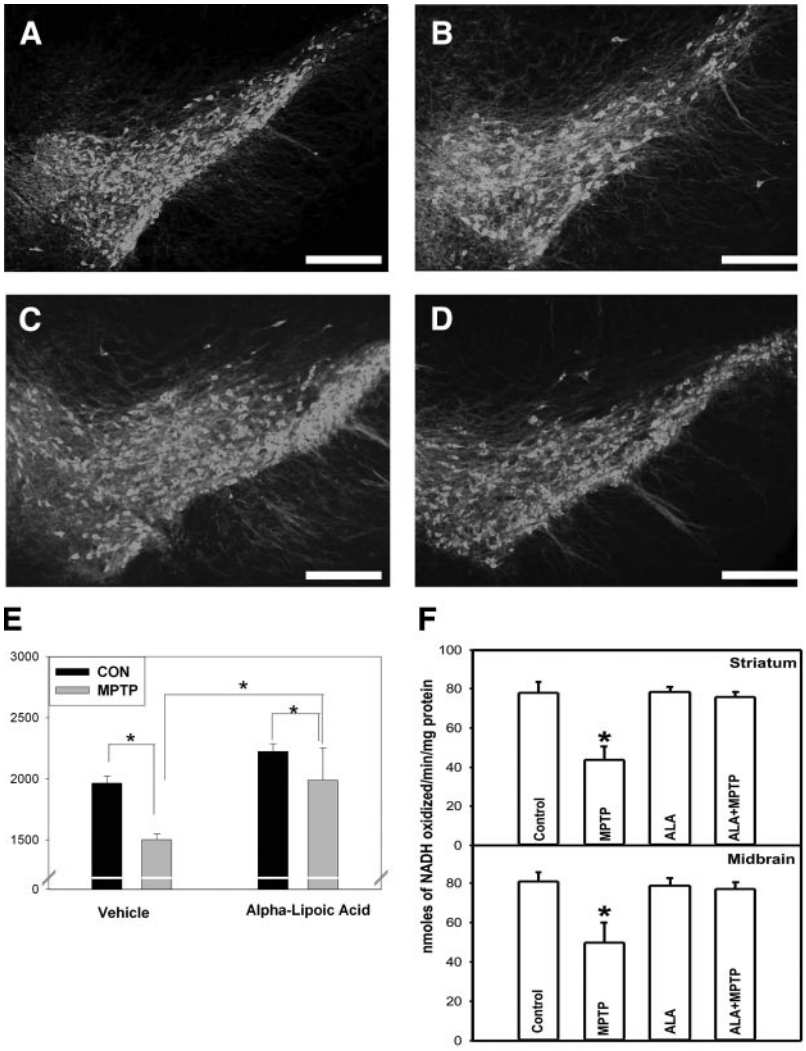

Figure 7. α-Lipoic acid attentuates MPTP-induced loss of dopaminergic neurons in SNpc and abolishes MPTP-mediated inhibition of complex I activity in mice. Tyrosine hydroxylase immunostaining of dopaminergic neurons in the substantia nigra of mice treated with vehicle (*A*), MPTP (*B*), ALA (*C*), or both ALA and MPTP (d) for 8 d as described above. Scale bar – 50 μm. *E)* Quantitative stereological analysis of the tyrosine hydroxylase positive neurons' profile from saline or ALA (*black bars*) and MPTP or ALA + MPTP (*gray bars*). Values are mean ± SEM (n=5). Asterisks indicate values significantly different from respective controls (P<0.01). Complex I activity was measured in the striatum and ventral midbrain (*F*). Complex I activity is expressed as nmoles of NADH oxidized/min/mg protein. Values are mean ± SD (n=6 animals) and asterisks indicate values significantly different from vehicle treated controls (P<0.002).

Exposure to MPP^+ resulted in translocation of Daxx, which was prevented by thiol antioxidants, such as N-acetyl cysteine (NAC) or ALA (Fig. 4). We then examined the cellular localization of Daxx in SNpc of mice treated with MPTP. Daxx translocated from nucleus to cytoplasm in SNpc neurons 24 h after a single dose of MPTP. Coadministration of ALA abrogated Daxx translocation to the cytosol. Further, in animals treated subchronically with MPTP for 8 d, a major portion of Daxx was seen in the cytosol in most of the surviving neurons in SNpc, while it was in the nucleus in majority of neurons in animals treated with both ALA and MPTP (Fig. 5). Further, the translocation of Daxx was seen predominantly in SNpc neurons that are affected by MPTP and in Parkinson's disease but not in the adjacent dopaminergic neurons in ventral tegmental area or in the substantia nigra pars reticulata that are unaffected by MPTP (Fig. 4). This is the first demonstration of Daxx translocation, *in vivo*, and the functional implications thereof. Daxx has been shown to translocate to the cytosol following exposure to β-amyloid peptide, *in vitro*, in cells, in a manner similar to that seen with hydrogen peroxide (36), but *in vivo* evidence has been lacking so far.

Note that after a single dose of MPTP, complex I activity recovers completely at 24 h (24). However, we find that Daxx continues to be present in the cytosol at this time point, indicating that the death pathway trigger persists longer after a single dose compared to the mitochondrial dysfunction measured as complex I activity. Further, after subchronic dosing with MPTP for 8 d, while ALA completely abolished complex I loss it was able to attenuate, but not abolish, dopaminergic cell loss. Presumably, ALA was unable to afford complete protection against cell death, indicating that a more potent antioxidant thiol was essential to completely block the MAPK death cascade.

MPP^+, the toxic metabolite of MPTP, directly inhibits complex I by binding to the rotenone binding site of complex I and also indirectly by generating oxidative stress. The recovery of complex I activity 24 h after MPTP is concordant with the absence of detectable MPP^+ in the ventral midbrain 12 h after MPTP (Fig. 8). Thus, it appears that MPP^+ is rapidly cleared from the ventral midbrain.

DJ-1 (PARK 7) is a redox-sensitive protein that has been implicated in certain familial forms of PD (37). DJ-1 has a redox sensitive cysteine residue (Cys106) that

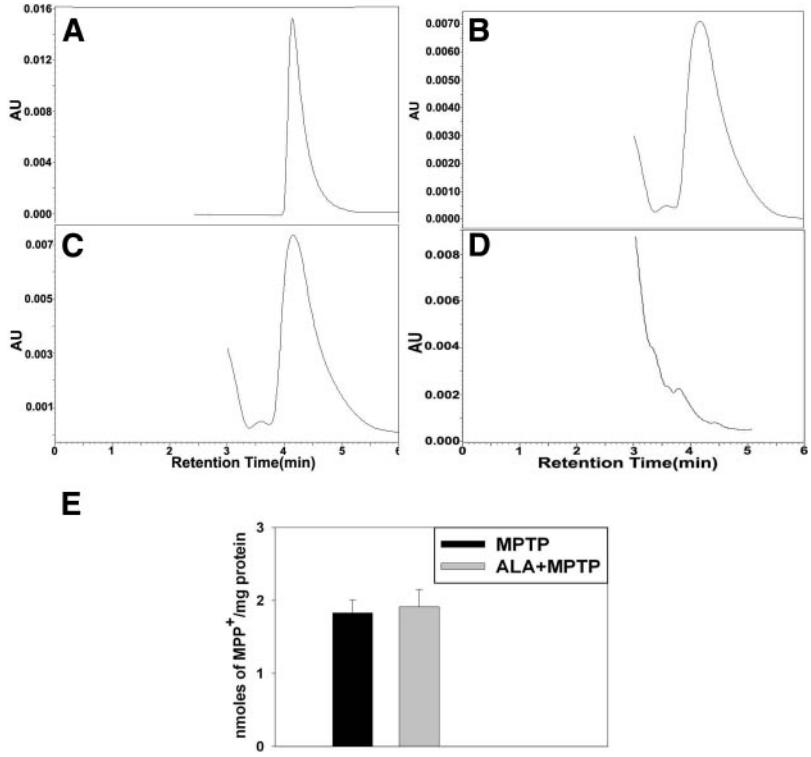

Figure 8. MPP^+ levels in ventral midbrain following MPTP administration. MPP^+ levels were measured in ventral midbrain following MPTP administration. HPLC chromatogram depicting standard MPP^+ (*A*), and MPP^+ levels 1 (*B*, *C*) and 12 h (*D*) after a single dose of MPTP. Some animals were also administered with sodium salt of α-lipoic acid (ALA; 22 mg/kg body weight, s.c.) 1 h prior to MPTP injection (*C*). MPP^+ concentration was similar in both the groups namely those treated with MPTP alone or with both ALA and MPTP (*E*; n=3).

is oxidatively modified following exposure to ROS (38). Although the role of Daxx in cell death is controversial, recent studies have shown that DJ-1 is associated with Daxx in the nucleus and this association prevents the translocation of Daxx to the cytosol (28). Mutations in DJ-1 that lead to familial Parkinson's disease prevent its association with Daxx in the nucleus. Here we find that MPTP exposure down-regulates DJ-1 levels, both in the nucleus and in the extranuclear compartments (Fig. 6). Thus, presumably the lowered levels of DJ-1 in the nucleus result in the dissociation of Daxx from DJ-1, leading to its translocation and association with ASK1 in the cytosol propagating the death cascade. This represents another mechanism of Daxx translocation in addition to that through its phosphorylation by JNK (39).

In this study, we demonstrate the potential of the thiol delivery agent, ALA to protect against the activation of apoptosis signaling pathway, the mitochondrial dysfunction and attentuate dopaminergic cell loss, *in vivo*, in mice. Although the protective effect of ALA has been described *in vitro*, in cultured cells (40), this is the first demonstration of its effect in an animal model of Parkinson's disease.

One of the primary effects of MPTP exposure is generation of oxidative stress leading to perturbation of thiol homeostasis seen as glutathione (GSH) loss and extensive modification of thiol groups in proteins. The lack of increase in glutathione disulphide (GSSG) levels following oxidative stress seems to indicate that the protein glutathione mixed disulfide (PrSSG) formation presumably occurs through formation of sulfenic acids as downstream effect of the oxidative stress generated by MPTP (Fig. 3*B*). Oxidative stress is a primary effect of MPTP, which can lead to dysfunction of complex I since complex I is extremely sensitive to the perturbation of thiol homeostasis (41). We down-regulated GSH levels using the inhibitor of cystathionine γ-lyase L-propargylglycine (PPG), since the commonly used inhibitor of γ-glutamyl-cysteine synthase, buthionine sulfoximine, does not cross the blood brain barrier. Cystathionine γ-lyase is the last enzyme of the transsulfuration pathway that synthesizes cysteine from methionine. Availability of cysteine is critical for GSH synthesis. PPG reduced GSH levels and inhibited complex I activity (Fig. 3*C*), which could be reversed by the disulfide reductant, dithiothreitol (8), indicating that oxidation of protein thiol groups are responsible for the loss of enzyme activity. These studies were initially performed in Swiss Albino mice, but since the C57BL6J mice are known to be more sensitive to MPTP-mediated cell loss, experiments that addressed cell death were performed using C57BL6J mice. We hypothesize that generation of oxidative stress leading to perturbation of protein thiols is a primary effect of MPTP, which is a causative factor not only for complex I dysfunction but can also trigger the activation of death pathway through activation of key signaling pathways triggered by redox perturbation, which are involved in the initiation of cell death cascade (**Fig. 9**).

Our studies demonstrate the activation of MAPK signaling cascade and translocation of Daxx in specific regions of the brain (namely ventral midbrain and striatum) following MPTP administration, *in vivo*, in mice. ALA prevents these effects, indicating that they are triggered by oxidative stress. Oxidative stress and the protein thiol modification resulting from MPTP exposure induce mitochondrial dysfunction via com-

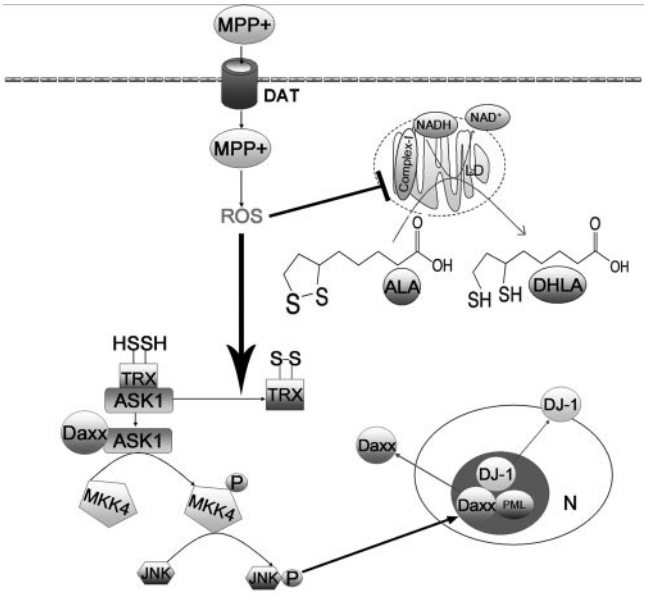

Figure 9. Schematic representation of the involvement of ASK1 and Daxx in MPTP mediated toxicity. 1-methyl-4-phenylpyridinium (MPP^+), the toxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes increased production of reactive oxygen species (ROS) and mitochondrial dysfunction in dopaminergic neurons by inhibiting complex I of the electron transport chain. ROS also activate apoptosis signal regulating kinase (ASK1) through the oxidation of thioredoxin (TRX), which helps its dissociation from ASK1. As a consequence, the downstream kinases MKK4 and Jun N-terminal kinase (JNK) are phosphorylated. Daxx subsequently translocates to the cytosol from the nucleus by dissociating from DJ-1 in the nucleus or through the phosphorylation of Daxx by JNK. The interaction of Daxx and ASK1 in the cytosol ensues the death cascade. Approaches aimed at maintenance of the protein thiol homeostasis through a thiol delivery agent, such as ALA can help terminate this cascade and attenuate MPTP-induced neurodegeneration. DAT—dopamine transporter. LD—lipoamide dehydrogenase. N—nucleus. PML—promyelocytic leukemia nuclear bodies. ALA—α-lipoic acid. DHLA—dihydrolipoic acid.

plex I inhibition and also trigger the MAPK pathway through activation of ASK1 (Fig. 9). Complex I inhibition can further generate oxidative stress propagating the cell death pathway. Thus, these effectors feed on each other presumably leading to the untimely demise of dopaminergic neurons. Thiol delivery agents offer an attractive option for terminating this cascade and protecting the further loss of dopaminergic neurons in Parkinson's disease. FJ

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