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Research Report

Constitutive expression and functional characterization of mitochondrial glutaredoxin (Grx2) in mouse and human brain

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ABSTRACT

Oxidative stress and mitochondrial dysfunction caused by loss of complex I activity are presumed to be primary events leading to neurodegeneration in Parkinson's disease. Mitochondrial glutaredoxin (Grx2), a glutathione-dependent thiol disulfide oxidoreductase helps maintain redox homeostasis in the mitochondria. We therefore, examined the constitutive expression of Grx2 in brain and its role in MPTP-mediated mitochondrial dysfunction in the extrapyramidal system. Grx2 is constitutively expressed in both neuron and glia in mouse and human brain including the neurons in human substantia nigra. Grx2 mRNA and protein were transiently upregulated in midbrain and striatum 1 h but not 4 h after a single dose of MPTP. Downregulation of Grx2 using antisense oligonucleotides, in vivo, in mouse brain resulted in partial loss of complex I activity indicating that Grx2 may help maintain complex I function in the mitochondria. Further, overexpression of Grx2 abolished MPP^+-mediated toxicity in vitro in neuroblastoma cells. Our results demonstrate the probable role of Grx2 in maintenance of the redox milieu in mitochondria and its potential neuroprotective role in preserving mitochondrial integrity in neurodegenerative diseases, such as Parkinson's disease.

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1. Introduction

Oxidative modification of cellular macromolecules and reduced levels of antioxidants have been detected in the brains of Parkinson's disease patients specifically in the substantia nigra (Jenner, 1998). Further, there is increasing evidence for involvement of mitochondria in neurodegenerative diseases including Parkinson's disease (PD; Park et al., 2006; Ohta and Ikuroh Ohsawa, 2006), wherein complex I dysfunction has been identified in mitochondria from platelet, brain and muscle of Parkinson's disease patients (Parker et al., 1989; Mizuno et al., 1995). Selective loss of GSH, which precedes the inhibition of mitochondrial complex I activity, has been reported to occur not only in PD (Perry et al., 1982) but also in animal models of PD (Sriram et al., 1997). A major consequence of oxidative stress in brain mitochondria following loss of glutathione (GSH) is the formation of protein–glutathione mixed disulfides leading to modification of proteins rather than increase in the levels of oxidized glutathione (GSSG) as seen in liver mitochondria (Ravindranath and Reed, 1990). Thus, maintenance of mitochondrial function during

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Abbreviations: DAPI, 4′,6′-diamidino-2-phenylindole; Grx2, mitochondrial glutaredoxin 2; MPTP, 1-methyl-4-phenyl-1,2,3,6,tetrahydropyridine; PD, Parkinson's disease

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oxidative stress would require the presence of an efficient system that can reduce protein disulfides to active thiols.

In mammalian cells, thiol homeostasis is maintained predominantly by thioredoxin and glutaredoxin systems. Glutaredoxins belong to a family of small proteins having thiol disulfide oxidoreductase activity and are present both in the cytosol (Grx1) and mitochondria (Grx2; Gladyshev et al., 2001). The cytosolic and mitochondrial glutaredoxin share about 36% sequence identity (Lundberg et al., 2001). Grx2 has a predicted size of 18 kDa and contains the active site sequence

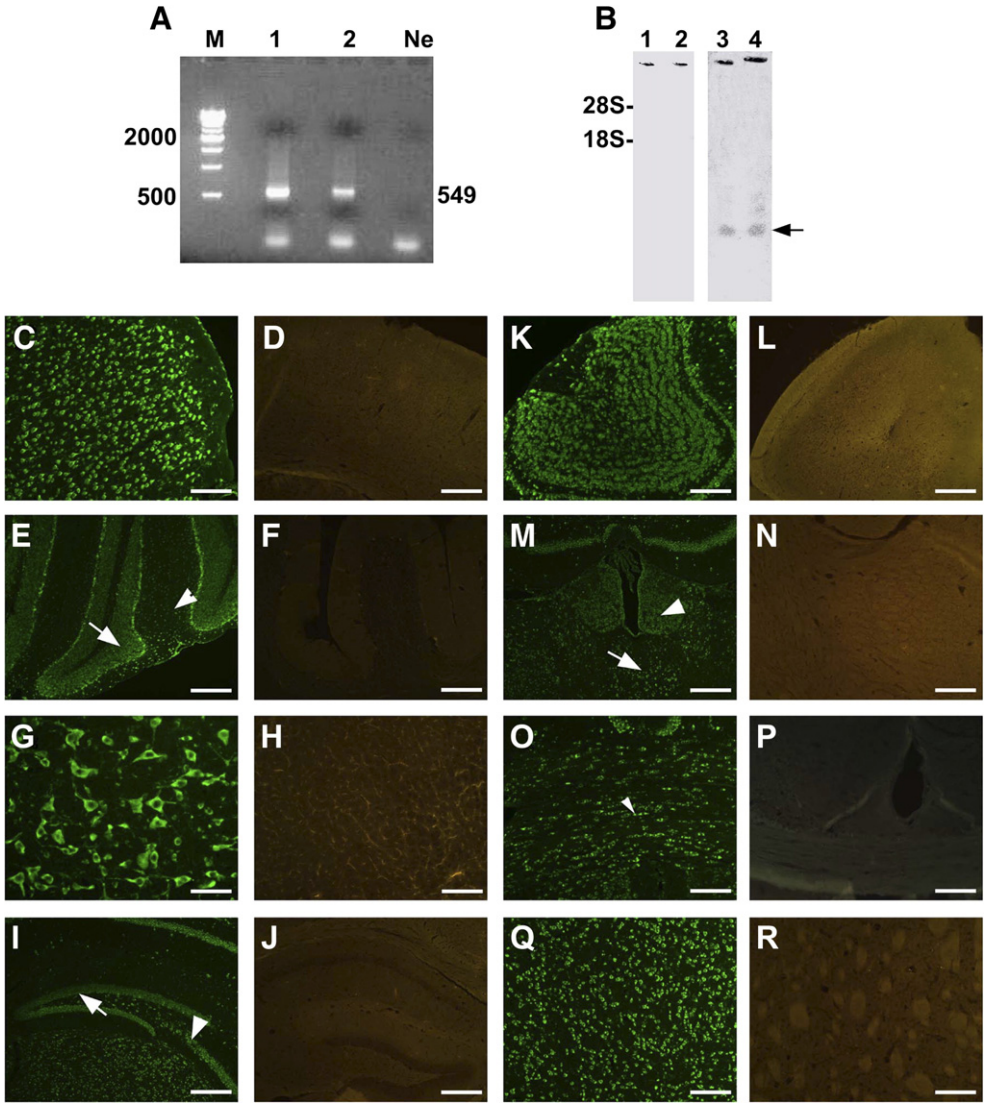


Fig. 1 – Constitutive expression and localization of Grx2 in mouse brain. The complete ORF of Grx2 was amplified from mouse brain using RT-PCR (A). An amplicon of 549 bp was generated (Lanes 1 and 2) 'M' indicates molecular weight marker and 'Ne' indicates negative control. (B) Total RNA from mouse brain (lanes 1 and 3, 15 μg; and lanes 2 and 4, 30 μg) was electrophoresed and hybridized with sense (lanes1 and 2) and antisense riboprobes (lanes 3 and 4) for Grx2. The mobility of the 18S and 28S ribosomal RNA is indicated. The expression of Grx2 in mouse brain is seen as a band corresponding to 500 bp. (C) In situ hybridization of coronal sections from mouse brain showing the presence of Grx2 mRNA in the neurons of cerebral cortex. Note the differential fluorescence in the laminar architecture of the cortex. Corresponding control section hybridized with the sense probe do not show any fluorescence (D). Intense fluorescent labeling of the Purkinje cells in the mouse cerebellum which express Grx2 mRNA (E). The inner granular layer (arrow) of the cerebellum was more intensely labeled than the molecular layer (arrowhead). The reticular neurons of the midbrain expressed Grx2 mRNA (G). Intense fluorescence was seen in the pyramidal cell layer and the granule cells of dentate gyrus (arrow) in the hippocampus. The presence of Grx2 mRNA in the pyramidal neurons of CA3 subfield in the hippocampus is shown (arrowhead; I). Grx2 expression was seen in the olfactory neurons (K). The habenular nucleus (arrowhead) and the paraventricular nucleus of the thalamus (arrow) expressed Grx2 mRNA (M). Glial cells of the corpus callosum (arrowhead) were also intensely fluorescent (O). The neurons in the caudate putamen are intensely stained (Q). Corresponding control sections hybridized with the sense probe are depicted as (F, H, J, L, N, P, R). Scale bar indicates 100 μm except in panels C, K, O, P, Q, R (scale bar=50 μm) and G (scale bar=25 μm).

"CSYC", which is different from the conserved "CPYC" motif present in Grx1. It is present both in the nucleus and mitochondria depending on whether transcription starts from the first or second ATG start codon (Lundberg et al., 2001). Grx2 protects against oxidative stress induced by mitochondrial damage (Daily et al., 2001a,b) and siRNA-mediated knockdown of Grx2 sensitizes HeLa cells to cell death induced by doxorubicin/adriamycin and phenylarsine oxide (Lillig et al., 2004). Further, the overexpression of Grx2 decreased the susceptibility to apoptosis (Enoksson et al., 2005). The role of Grx2 in maintaining the redox milieu of mitochondria during oxidative stress has been studied in cultured cells. However, the cell culture models have unavoidable limitations and animal studies are critical in determining the central role played by glutaredoxin in the mitochondria.

MPTP mediated mitochondrial dysfunction plays a major role in the degeneration of substantia nigra pars compacta neurons in animal models (Nicklas et al., 1985). Using the MPTP mouse model of PD, we have shown the role of cytosolic Grx1 in maintaining complex I activity in the mitochondria. Down-regulation of cytosolic glutaredoxin using antisense oligonucleotides prevented the recovery of complex I in the striatum after MPTP treatment, providing support for the critical role of glutaredoxin in the recovery of mitochondrial function in brain (Kenchappa and Ravindranath, 2003). Complex I contains several cysteine residues in its active site (Dupuis et al., 1991), and the facile glutathionylation of thiol groups in the enzyme by reactive oxygen species can potentially result in inhibition of the enzyme. Our earlier studies show that the complex I inhibition caused by MPTP can be reversed by dithiothreitol, further confirming that protein thiol oxidation is a primary cause for complex I inhibition (Annepu and Ravindranath, 2000). Therefore, maintenance of protein thiol homeostasis is critical for preserving the functional activity of complex I.

Grx2 de-glutathionylates mitochondrial proteins, such as complex I, which form mixed disulfides during oxidative conditions in bovine heart mitochondria (Beer et al., 2004). However, little is known about the interplay between the mitochondrial and cytosolic glutaredoxin in maintaining the integrity and functionality of mitochondrial complex I, *in vivo*. We therefore studied the constitutive expression and localization of Grx2 in mouse and human brain and examined its functional role in MPTP-mediated mitochondrial toxicity in mice.

2. Results

2.1. *Amplification of the open reading frame of Grx2 from mouse brain and its localization*

We amplified the complete ORF of Grx2 by RT-PCR using total RNA from mouse brain (Fig. 1A). The sequencing of the RT-PCR product revealed complete sequence identity with mouse Grx2. The sequence had 86% homology to human Grx2. Northern blot analysis of total RNA from mouse brain cortex provided further evidence for its constitutive expression in mouse brain (Fig. 1B). Grx2 was localized in mouse brain using fluorescent *in situ* hybridization (FISH). In mouse brain, Grx2 mRNA was localized in the neurons of cerebral cortex (Fig. 1C), Purkinje cells of the cerebellum (Fig. 1E) and reticular neurons of midbrain (Fig. 1G). Intense fluorescence was seen in the hippocampus, in the pyramidal neurons of CA1, CA2 and CA3 subfields and in the granule cell layer of dentate gyrus (Fig. 1I) and in the olfactory bulb (Fig. 1K). The habenular and paraventricular nucleus of the thalamus (Fig. 1M) and striatum (Fig. 1Q) showed distinct Grx2 localization. In the corpus callosum, the glial cells were stained intensely (Fig. 1O). The respective controls hybridized using sense probe are also depicted (Figs. 1D, F, H, J, L, N, P, R).

2.2. *MPTP exposure upregulates Grx2 in midbrain and striatum*

The constitutive expression of Grx2 protein was detected by immunoblot in the mitochondria but not in the cytosol (Fig. 2A). The specificity of the antiserum was also examined by immunostaining the cells preloaded with MitoTracker with antibody to Grx2. Grx2 immunostaining colocalized with that of MitoTracker indicating that Grx2 was present in the mitochondria (Fig. 2B). As anticipated, no Grx2 was detectable in the cytosol (Fig. 2A). Male mice were administrated a single dose of MPTP (30 mg/kg body weight, s.c.) and sacrificed 1 and 4 h later ($n=3$ animals for each time point). Grx2 protein increased significantly at 1 h following MPTP but was not significantly different at 4 h, both in midbrain and striatum (Fig. 2C). The increase in protein level was due to transcriptional activation of Grx2 since the mRNA levels were also increased nearly 2-fold in the midbrain after 1 h of MPTP treatment (Fig. 2D).

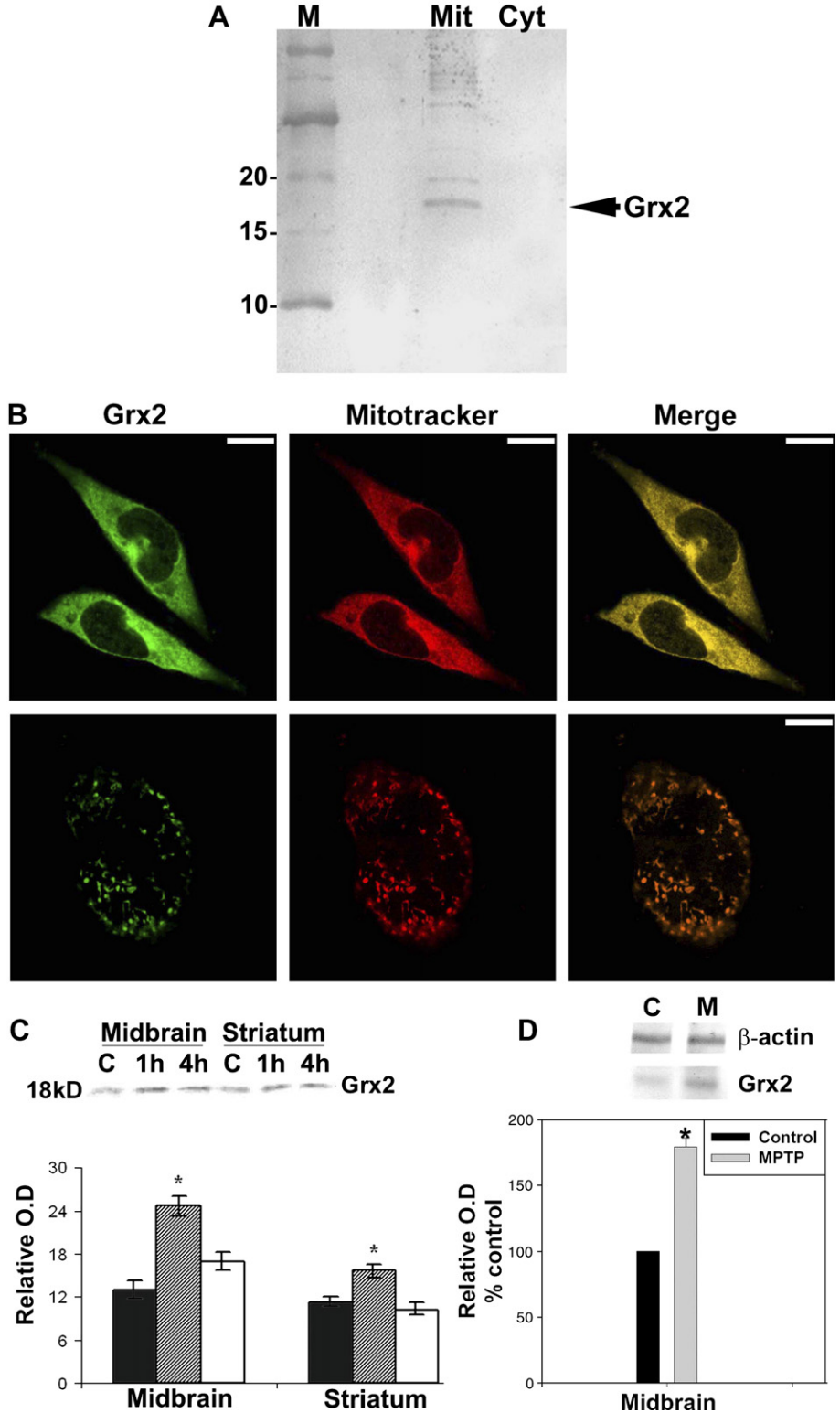
Fig. 2 – Effect of MPTP on Grx2 mRNA and protein levels. (A) Representative immunoblot of mitochondrial (Mit) and cytosolic (Cyt) fractions prepared from whole brain homogenate immunostained with antiserum to Grx2 is depicted. (B) Immunocytochemical detection of Grx2 is depicted in green, while staining with MitoTracker is shown in red in Neuro2A cells. MitoTracker staining colocalizes with Grx2 indicating the specificity of the antiserum used (scale bar = 10 μm). The lower panel shows a confocal image of a cell with the mitochondria highlighted using MitoTracker which colocalizes with Grx2 (scale bar = 5 μm). (C) Mice were sacrificed 1 and 4 h after a single dose of MPTP. A representative blot from midbrain and striatum of control and MPTP-treated animals immunostained with antiserum to Grx2 is depicted. Lanes contained 10 μg of mitochondrial protein. Densitometric analysis of the immunoblots representing the relative intensity of the immunoreactive bands from control (dark grey bar), 1 (grey bar) and 4 h (empty bar) MPTP-treated animals. Values are mean ± SD ($n=3$ animals). Asterisks indicate values significantly different from corresponding control ($p<0.05$). (D) Expression of Grx2 mRNA was increased in midbrain following 1 h of MPTP treatment. A representative northern blot of Grx2 and β-actin from midbrain of vehicle (C) and MPTP-treated mice (M; $n=3$ for each time point). Densitometric analysis of northern blots depicting the relative intensity of the hybridized bands are represented.

2.3. *Effect of downregulation of Grx2*

We downregulated Grx2 in brain by intrathecal administration of antisense oligonucleotides (Fig. 3A; $n=3$ animals for each time point). Downregulation of Grx2, per se, caused a small but significant loss (14% decrease) in complex I activity in the midbrain but the complex I loss in striatum was more pronounced (28% loss; Fig. 3B).

2.4. *Overexpression of Grx2 abolishes MPP^+-mediated toxicity in Neuro2a cells*

Neuro2a cells were transfected with pCMV-Grx2 (Figs. 4A and B) or empty vector (control) and then exposed to MPP^+ (1 mM) for 24 h. Apoptotic cell death was assessed using the terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL). Cells transfected with empty vector and



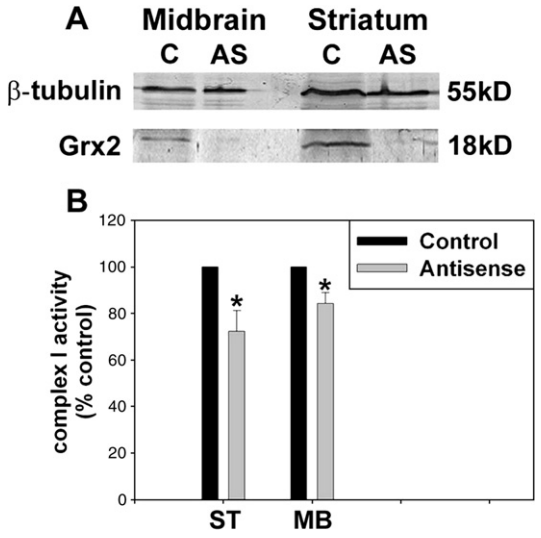


Fig. 3 – Effect of downregulation of Grx2 on complex I loss. (A) Mice were administered antisense oligonucleotides (100 μg, twice at 12 h interval) to Grx2 through intrathecal route and sacrificed 12 h after the last injection. Immunoblot analysis of Grx2 in midbrain and striatum from control (C) and antisense (AS) oligonucleotide-treated mice reveals that antisense oligonucleotides to Grx2 downregulate Grx2 expression. (B) Complex I activity in midbrain and striatum of animals treated with antisense oligonucleotides (as described above). The enzyme activity is expressed as nanomoles of NADH oxidized/min/mg protein. Complex I activity in midbrain of control animals was 55.017 ± 4.835 and antisense oligonucleotide-treated animals was 46.667 ± 6.506. Complex I activity in striatum of control animals was 64.303 ± 2.837 and the antisense oligonucleotide-treated animals was 46.667 ± 6.110. Values are mean ± SD ($n = 3$) animals. Asterisks indicate values significantly different from vehicle-treated control ($p < 0.05$).

exposed to MPP^+ showed significantly higher TUNEL immunoreactivity (37 ± 5.7% of cells) as compared to cells not exposed to MPP^+ (7.7 ± 2.5%). Grx2 overexpressing cells exposed to MPP^+ revealed less TUNEL immunoreactivity (11.7 ± 3.2%; Figs. 4C and D) indicating the lowered levels of apoptosis as compared to control cells exposed to MPP^+. Grx2 overexpressing cells (6.8 ± 4.5%) had similar number of apoptotic nuclei as cells transfected with empty vector.

2.5. *Constitutive expression of Grx2 in human brain*

Northern blot analysis demonstrated the constitutive expression of Grx2 in human brain (Fig. 5A). Further, localization of Grx2 mRNA using FISH demonstrated the expression of Grx2 across human brain regions. High levels of Grx2 mRNA were seen in cerebral cortex (Fig. 5C), midbrain (Fig. 5G) and hippocampus (Fig. 5I), while sections hybridized with the sense probe showed no fluorescence (Figs. 5D, F, H, J). In the cerebellum, the Purkinje cells and granular cell layer showed intense fluorescence (Fig. 5E), while the molecular cell layer showed sparse staining. Intense fluorescence was seen in pyramidal neurons of CA1, CA2 (data not shown) and CA3 regions of the hippocampus. In the midbrain, the reticular neurons were selectively labeled indicating the predominant presence of Grx2 mRNA in these cell populations.

Neurons in the substantia nigra from human brain, which exhibit dopaminergic phenotype, expressed Grx2 (Fisg. 6A and B) as examined by immunohistochemistry while the control sections stained with non-immune serum did not show significant staining (Figs. 6C and D). In mouse brain substantia nigra Grx2 expressing cells (Fig. 6E) colocalized with tyrosine hydroxylase positive (Fig. 6F) dopaminergic neurons as depicted in the merged figure (Figs. 6G and H).

3. Discussion

Mitochondrial glutaredoxin 2 (Grx2) is a glutathione-dependent oxidoreductase with an active site consisting of Cys-Ser-Tyr-Cys that helps maintain redox homeostasis in the mitochondria. The four cysteine residues present in 2 molecules of Grx2 are coordinated with iron in a non oxidizable $[2Fe–2S]^{2+}$ cluster to form dimeric holo Grx2, which is inactive (Lillig et al., 2005). The cluster is preserved in the presence of glutathione, while oxidants such as glutathione disulfide promote the degradation of the dimer leading to the formation of the Grx2 monomer, which results in activation of the enzyme. The iron–sulfur cluster thus serves as a redox sensor and activates Grx2 during oxidative stress. We examined the presence of Grx2 in mouse and human brain and studied its potential role in maintenance of mitochondrial function by downregulating Grx2 mRNA, *in vivo* using antisense oligonucleotides.

The mouse and human Grx2 shared only 86% homology (Supplementary Fig. 1); therefore, we first cloned mouse Grx2 by RT-PCR amplification of mouse brain RNA (Fig. 1A). Our experiment demonstrated that Grx2 is expressed ubiquitously in both mouse and human brain and is localized in both neuronal and glial cell populations. Importantly, Grx2 could be localized in the melanized neurons of the substantia nigra, the very cells that degenerate in Parkinson's disease (Figs. 6A, B). Interestingly while we observed the preferential localization of Grx1 mRNA in neuronal cell population (Balijepalli et al., 2000), in the present study we note that Grx2 is distributed more ubiquitously in the brain in both neuronal and glial cell populations.

We observed transient upregulation of Grx2 mRNA and protein 1 h following MPTP administration (Figs. 2C, D). This could be due to stress response following the initial oxidative stress caused by MPTP. Grx2 is known to be upregulated in yeast *Saccharomyces cerevisiae* following oxidative insult (Grant et al., 2000). Here we demonstrate for the first time a similar upregulation in mammalian brain following MPTP exposure. Promoter analysis of mouse Grx2 reveals the presence of an AP1 site (1743 bp upstream of transcription start site) and NFκB binding sites (1780,1781,667,666 bp upstream of transcription start site). Presumably, activation of AP1 or NFκB that is shown to occur following MPTP administration (Kenchappa and Ravindranath, 2003) could mediate the upregulation of Grx2 seen after MPTP administration.

We downregulated Grx2 in mouse brain using antisense oligonucleotides to examine the role of Grx2 in maintenance

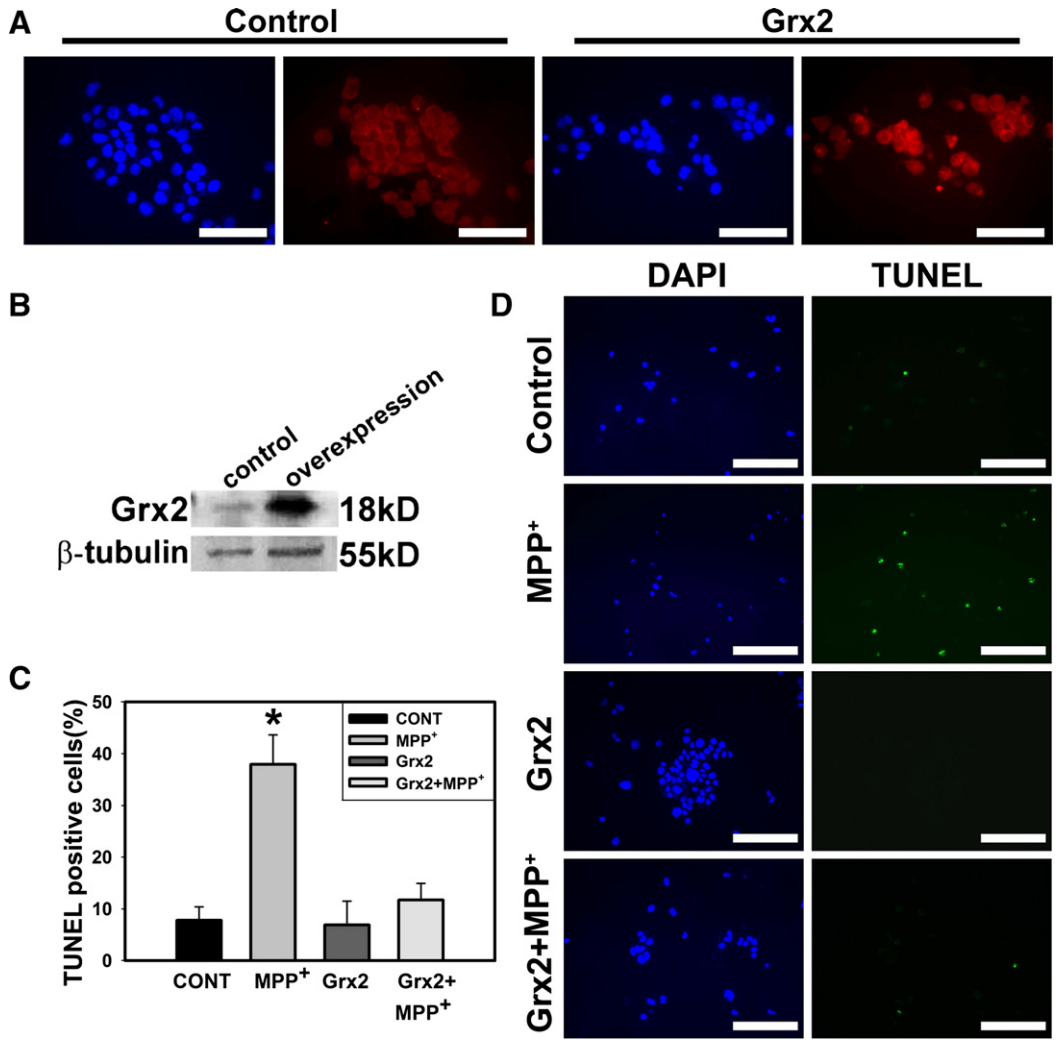


Fig. 4 – Overexpression of Grx2 abolishes MPP⁺-mediated toxicity in Neuro2a cells. (A) Immunostaining for Grx2 in N2A cells transfected with empty vector (pCMV-MCS; Control) or with pCMV-MCS containing the complete ORF of Grx2 (Grx2) is shown along with respective DAPI images (scale bar = 100 μM). (B) Representative immunoblot stained with Grx2 antiserum of control and Grx2 overexpressing cells. (C) Cells were exposed to MPP⁺ (1 mM) for 24 h and the percentage of TUNEL-positive cells vs. DAPI-stained nuclei were counted from 3 independent experiments. Asterisk indicates values significantly different from controls ($p < 0.001$). Images of TUNEL positive cells along with corresponding DAPI images are depicted (scale bar = 200 μM).

of complex I activity. Downregulation of Grx2, per se, caused reduction in complex I activity in both striatum and midbrain (Fig. 3B). This may be expected since oxidation of critical subunits in complex I (75 and 52 kDa) through thiol modification is known to result in the loss of complex I activity and Grx2 helps restore complex I activity *in vitro* in isolated mitochondria (Taylor et al., 2003). It may be noted that although Grx2 knockdown was substantial, the loss in complex I activity was only 14%. This may be due to the fact that both Grx1 and Grx2 contribute to the maintenance of complex I activity. We have earlier shown that knockdown of Grx1 also results in complex I loss (Kenchappa and Ravindranath, 2003) and it is presumable that when Grx2 is downregulated compensatory mechanisms involving Grx1, thioredoxins (Trx1 and Trx2) and peroxiredoxins may potentially contribute to the restoration of complex I activity.

Overexpression of Grx2 in Neuro2a cells completely ameliorated the apoptotic cell death mediated by MPP⁺ (Fig. 4), indicating the potential neuroprotective effects of Grx2. Thus, Grx2 is important for the maintenance of complex I activity, *in vivo* in brain and therefore may play an important role in ensuring mitochondrial integrity in vulnerable neurons, such as dopaminergic neurons of the substantia nigra pars compacta that degenerate in PD. Single nucleotide polymorphisms that alter the functional activity of Grx2 may render individuals vulnerable to complex I dysfunction such as those seen in PD.

4. Experimental procedure

4.1. *Materials*

cDNA to human mitochondrial glutaredoxin (Grx2) was kindly provided by Dr. Vadim N. Gladyshev, University of Nebraska, Lincoln (Gladyshev et al., 2001). DIG-RNA labeling and

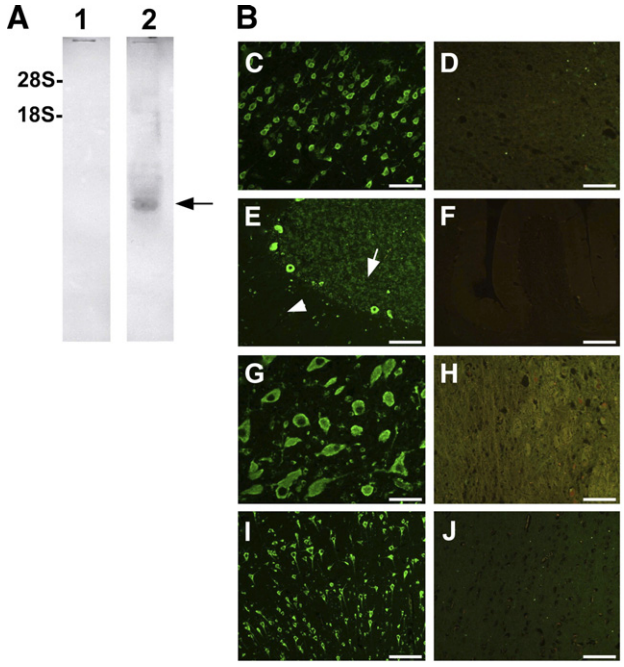


Fig. 5 – Constitutive expression and localization of Grx2 in human brain. (A) Northern blot analysis showing the constitutive expression of Grx2 mRNA in human brain. Total RNA from human brain cortex (lanes1, 2; 15 μg) was electrophoresed and hybridized with sense (lane1) and antisense riboprobes (lane 2) prepared using cDNA to human Grx2. The mobility of the 18S and 28S ribosomal RNA are indicated. The expression of Grx2 in human brain was seen as band of 500 bp. (B) Localization of Grx2 mRNA in human brain using fluorescent in situ hybridization. The presence of Grx2 mRNA was seen in the neurons of cerebral cortex (C). Intense fluorescence was seen in Purkinje and granule cells (arrow) of cerebellum. Sparse staining was observed in the molecular layer (arrowhead; E). The reticular neurons in the midbrain expressed Grx2 mRNA (G). Intense fluorescence was seen in the pyramidal cell layer of CA3 region of the hippocampus (I). Corresponding controls hybridized with the sense probe are depicted in panels D, F, H, J. Scale bar indicates 50 μm except in panels F (scale bar=100 μm) and G, D (scale bar=25 μm).

detection kit, antidigoxigenin Fab fragments linked to peroxidase and alkaline phosphatase were purchased from Roche Biochemicals (Indianapolis, IN, USA). The tyramide signal amplification kit was obtained from New England Nuclear (Boston, MA, USA) and 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 Chemical Company (St. Louis, MO, USA) or Qualigens (India).

4.2. *Animals*

All animal experiments were carried out as per the National Institute of Health 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 mice (2 to 3 months, 25–30 g) were obtained from the Central Animal Research Facility of National Brain Research Centre (NBRC). Animals had access to pelleted diet and water *ad libitum*.

4.3. *Human brain samples*

Brain tissue from male traffic accident victims were obtained from the Human Brain Tissue Repository of the National Institute of Mental Health and Neurosciences, Bangalore, India, in compliance with the ethical guidelines of the Government of India. After autopsy, brains were washed in ice-cold saline and dissected into different regions based on standard anatomical markings including cortex, hippocampus, midbrain and cerebellum. All regions were fixed in buffered paraformaldehyde (4%, w/v) for *in situ* hybridization studies. For northern blot analysis, brain samples were flash frozen in liquid nitrogen and stored at −70 °C. The age and gender of the subjects were: 32 year old male (postmortem delay 4 h) and 65 year old male (postmortem delay 6 h).

Animals were administered a single dose of MPTP (30 mg/kg body wt in normal saline, s.c.). Control animals received vehicle alone. Animals were sacrificed at various times after MPTP administration. The mice were anesthetized with ether and perfused transcardially with ice-cold normal saline before decapitation. Striatum was dissected out as described by Glownisky and Iversen (1966). Bregma coordinates and anatomic boundaries were established for dissection of ventral midbrain based on the mouse brain atlas (Paxinos and Franklin, 2001). 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. The slice was placed rostral side up and the ventral midbrain was dissected out ensuring that there was no contamination of the hippocampus, cortex or cerebellum. The dissected brain regions were immediately frozen in liquid nitrogen.

4.4. *Amplification of Grx2 by RT-PCR using mouse brain RNA*

Total RNA was isolated from mouse brain using TRI reagent (Chomczynski, 1993). One-step RT-PCR was performed according to the manufacturer's protocol (Qiagen, GmbH, Germany) using the following forward and reverse primers; 5′ to 3′-AGCTGCCGGGGACCTTTG (representing bases from 16 to 33) and 3′ to 5′-GTCAAAGGTACGACTGCACTA (representing bases from 484 to 504). The primers were designed to amplify the coding sequence starting from the mitochondrial translocation signal of exon 1. The expected size of the PCR product is 549 bp. Negative control was devoid of template DNA. The PCR products were analyzed by agarose gel electrophoresis and cloned into pCR 2.1 vector (Invitrogen). Identity of the product was confirmed by sequencing. The Grx2 gene was further subcloned into pBluescript and sequence verified. The cDNA of human Grx2 present in pET vector was subcloned into pBluescript vector and verified by sequencing. These clones were used to synthesize sense and antisense riboprobes for northern blot analysis and *in situ* hybridization.

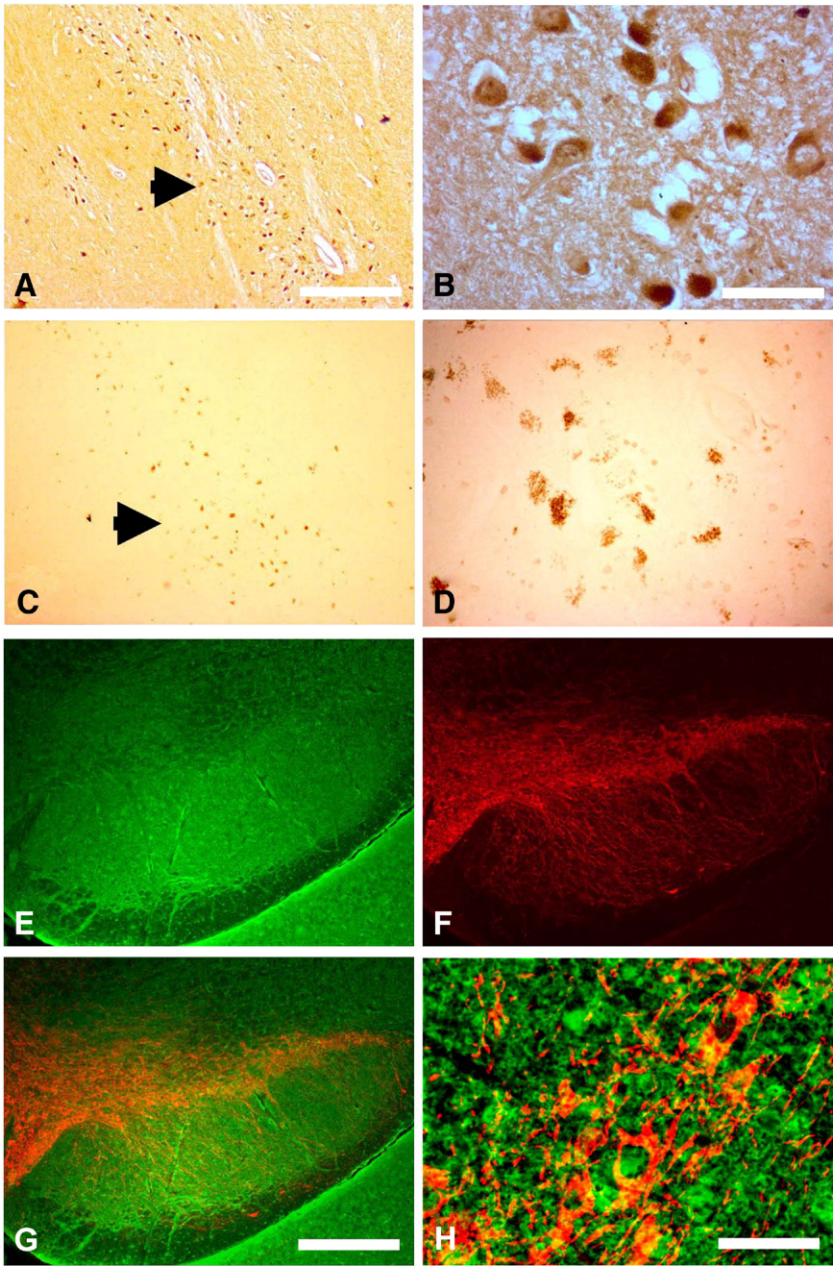


Fig. 6 – Localization of Grx2 in human and mouse brain substantia nigra by immunohistochemistry. Grx2 is expressed in substantia nigra (arrow; A) of human brain as seen by immunohistochemistry using antiserum to Grx2. A higher magnification of panel A shows prominent immunostaining in nigral neurons (B). The respective control section treated with non-immune serum (C) and its magnified image (D) are depicted. Arrow indicates the melanin laden dopaminergic pars compacta population of cells (C). Scale bar = 100 μm (A, C) and scale bar = 25 μm (B, D). Immunostaining of mouse brain substantia nigra with antisera to tyrosine hydroxylase (F) and Grx2 (E) is depicted. The merged image indicates the colocalization of Grx2 and tyrosine hydroxylase in substantia nigra (G and H). Scale bar = 100 μm (E–G) and scale bar = 5 μm (H).

4.5. *Processing of tissue*

Brain regions were homogenized in 0.25 M sucrose and centrifuged at 1000×*g* for 10 min to obtain the nuclear pellet and post-nuclear supernatant which was again centrifuged at 17,000×*g* for 30 min to obtain mitochondrial pellet. The mitochondrial pellet was suspended in 0.25 M sucrose and freeze–thawed for the assay of complex I and immunoblot analyses. Cytosol was prepared by ultracentrifugation of the homogenate at 65,000 rpm for 1 h. Protein concentration was estimated by a dye binding method (Bradford, 1976).

4.6. *Northern blot analysis and fluorescent in situ hybridization*

Constitutive expression and localization of Grx2 was examined by northern blot analysis (Kevil et al., 1997) and fluorescent *in situ* hybridization (Balijepalli et al., 2000), respectively,

using digoxigenin labeled sense and antisense riboprobes prepared using the cDNA to mouse and human brain Grx2.

4.7. *Preparation of antiserum to Grx2*

Antiserum to Grx2 was raised in New Zealand white rabbits (Kamataki et al., 1976). The 14 amino acid peptide (CNYKV-VELBLLEYG) was conjugated with bovine serum albumin at the N-terminal and used as antigen. This peptide sequence shared 93% homology with human Grx2 (NYKVVELDLLEYG) and 79% homology with mouse Grx2 (NYKAVELDMLEYG). The antiserum was used for immunoblotting and immunohistochemistry of Grx2 of both mouse and human origin.

4.8. *Immunoblot analysis*

Immunoblot analysis was performed using mitochondria from midbrain of vehicle and MPTP-treated mice. Proteins were transferred to nitrocellulose membranes (Towbin et al., 1979), incubated with antibody to Grx2 followed by incubation with anti-rabbit IgG labeled with alkaline phosphatase. Immunostained bands were detected using nitroblue tetrazolium and 5-bromo 4-chloro 3-indolyl phosphate as chromogens or using chemiluminescence kit (ECL, Amersham Pharmacia Biotech, France).

4.9. *Immunohistochemistry*

Human brain obtained at autopsy was fixed in 4% (w/v) buffered paraformaldehyde, processed for paraffin embedding and serial sections (8–10 μm thick) were cut. Sections were dewaxed, hydrated in graded alcohol and PBS (10 mM, pH 7.4, PBS). The sections were blocked with normal goat serum for 30 min at room temperature and incubated with antiserum to Grx2 (diluted to 1:1000 in PBS) at 37 °C for 2 h. After washing, they were incubated in biotinylated anti-rabbit IgG solution (1:500 dilution) for 1 h at room temperature. After further washing, they were incubated with VECTASTAIN Elite ABC reagent for 30 min at room temperature. The sections were then washed briefly in PBS followed by water. The color was developed using diaminobenzidine and mounted using Permont (Chinta et al., 2002). The same procedure was followed for the negative control except for the primary antibody was substituted for normal rabbit IgG. In a separate set of experiments, immunostaining was visualized using FITC labeled secondary antibody and the sections were counterstained with DAPI.

Neuro 2a cells were seeded in a chamber slide to 50% confluency 24 h before fixation. Mitochondria were stained with MitoTracker Deep Red 633 (Molecular Probes) at 37 °C prior to fixation with 4% paraformaldehyde at room temperature for 30 min. For immunostaining with Grx2, cells were washed with PBS and permeabilized with digitonin (0.01%; w/v) followed by overnight incubation with antisera to Grx2 at 4 °C. After washing with PBS, cells were incubated with secondary antibody (FITC tagged) for 1 h at room temperature. Finally the cells were mounted in mounting medium containing DAPI. Images were taken using confocal microscope.

4.10. *Overexpression of Grx2 and MPP^+ toxicity*

Neuro2a cells were plated in chamber slide at a cell density of 2×10^4 cells per well 24 h prior to transfection with pCMV-MCS (empty vector as control) and pCMV-Grx2 containing the complete coding sequence of mouse Grx2. After 24 h of transfection, cells were exposed to MPP^+ (1 mM) or vehicle for a further period of 24 h following which they were fixed with 4% paraformaldehyde (w/v). The cells were then permeabilized with 0.02% (v/v) Triton-X 100 and the TUNEL reaction was carried out using "*In situ* cell death detection kit, fluorescein" (Roche Indianapolis, IN) according to manufacturer's instruction. The cells were then washed with PBS (1X) and DNA fragmentation was detected by labeling with fluorescein labeled dUTP using terminal deoxynucleotidyl transferase and examined using a fluorescence microscope at excitation wavelength of 488 nm. The total cell nuclei were counted by counterstaining with 4′, 6′-diamidino-2-phenylindole (DAPI) at excitation wavelength, 350 nm.

4.11. *Assay of NADH:ubiquinone oxidoreductase (Complex I)*

Complex I was assayed in mitochondria as rotenone-sensitive NADH-ubiquinone oxidoreductase (Kenchappa and Ravindranath, 2003). 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 0.02 ml of 5 mM NADH solution. Test and blank reactions were run separately using a single beam spectrophotometer. The rate of decrease of absorbance at 340 nm (0.04–0.09/min) was monitored over 3 min. The assay was also performed in the presence of rotenone to determine the rotenone-sensitive enzyme activity that was 80% of the total activity. The enzyme activity is expressed as nanomoles of NADH oxidized per min per mg of protein.

4.12. *Downregulation of Grx2 expression in mouse brain using antisense oligonucleotides*

Antisense phosphorothionate end-capped oligonucleotides (19 mer) originating from the start codon of Grx2 cDNA (antisense: ATGTCCTGGCGCCGCGCGG) were injected intrathecally into mice at 100 μg/dose, twice at 12 h intervals. The downregulation of Grx2 was examined by immunoblot analysis 12 h after the last injection of oligonucleotides. The striatum and midbrain of the animals were dissected out and complex I activity was assayed as described earlier.

Statistical analysis was performed using Student's *t* test or ANOVA followed by Student–Newman–Keuls or Dunnett's test as appropriate.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.brainres.2007.09.019.

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