Published in final edited form as:

Toxicol Lett. 2014 August 4; 228(3): 179–191. doi:10.1016/j.toxlet.2014.05.020.

Hydrogen Peroxide responsive miR153 targets Nrf2/ARE cytoprotection in paraquat induced dopaminergic neurotoxicity^a

Madhusudhanan Narasimhan^{#,†,*}, Amanjot Kaur Riar[#], Mary Latha Rathinam[#], Dhanashree Vedpathak[#], George Henderson^{#,†}, and Lenin Mahimainathan^{#,†,*}

[#]Department of Pharmacology and Neuroscience, Texas Tech University Health Sciences Center, Lubbock, Texas, USA

[†]South Plains Alcohol and Addiction Research Center, Texas Tech University Health Sciences Center, Lubbock, Texas, USA

Abstract

Epidemiological and animal studies suggest that environmental toxins including paraquat (PQ) increase the risk of developing Parkinson's disease (PD) by damaging nigrostriatal dopaminergic neurons. We previously showed that overexpression of a group of microRNAs (miRs) affects the antioxidant promoting factor, Nrf2 and related glutathione-redox homeostasis in SH-SY5Y dopaminergic neurons. Although, dysregulation of redox balance by PQ is well documented, the role for miRs and their impact have not been elucidated. In the current study we investigated whether PQ impairs Nrf2 and its related cytoprotective machinery by misexpression of specific fine tune miRs in SH-SY5Y neurons. Real time PCR analysis revealed that PQ significantly ($p < 0.05$) increased the expression of brain enriched miR153 with an associated decrease in Nrf2 and its function as revealed by decrease in 4× ARE activity and expression of GCLC and NQO1. Also, PQ and H₂O₂-induced decrease in Nrf2 3' UTR activity was restored on miR153 site mutation suggesting a 3' UTR interacting role. Overexpression of either anti-miR153 or Nrf2 cDNA devoid of 3' UTR prevented PQ and H₂O₂-induced loss in Nrf2 activity confirming that PQ could cause miR153 to bind to and target Nrf2 3' UTR thereby weakening the cellular antioxidant defense. Adenovirus mediated overexpression of cytoplasmic catalase (Ad cCAT) confirmed that PQ induced miR153 is hydrogen peroxide (H₂O₂) dependent. In addition, Ad cCAT significantly ($p < 0.05$) negated the PQ induced dysregulation of Nrf2 and function along with minimizing ROS, caspase 3/7 activation and neuronal death. Altogether, these results suggest a critical role for oxidant mediated miR153-Nrf2/ARE pathway interaction in paraquat neurotoxicity. This novel

^aPreliminary results from these studies were presented at the Lone Star Chapter of the Society of Toxicology (LSSOT) annual meeting, October 17-18, Lubbock, Texas, USA.

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*Address for Correspondence: Lenin Mahimainathan (or) Madhusudhanan Narasimhan, Department of Pharmacology & Neuroscience, Texas Tech University Health Sciences Center, 3601 4th Street, STOP 6592, Lubbock, Texas, 79430 USA. Tel: 806-743-2425; Fax: 806-743-2744, lenin.mahimainathan@ttuhsc.edu (Lenin Mahimainathan) (or) madhu.narasimhan@ttuhsc.edu (Madhusudhanan Narasimhan).

Conflict of interest statement: All authors declare that there are no conflicts of interest.

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finding facilitates the understanding of molecular mechanisms and to develop appropriate management alternatives to counteract PQ-induced neuronal pathogenesis.

Keywords

Paraquat; miR153; H₂O₂; Nrf2; neurotoxicity

1. Introduction

Herbicides and pesticides are becoming increasingly potent environmental threats and their usage has increased by 527 million pounds and 404 million pounds respectively from 1996 to 2011 (Benbrook, 2012). Importantly, exposures to these environmental toxins are not only restricted to occupational setting but can stretch out to general residential background (CDC, 2009). Among agricultural chemicals, paraquat (PQ) is a well known herbicide that chiefly affects dopamine systems causing severe pathophysiological alterations and irreversible injuries to dopaminergic neurons (Betarbet *et al.*, 2000; Peng *et al.*, 2004; Ramachandiran *et al.*, 2007). Of note, nigral dopaminergic neuronal loss is documented to be the reason for dysfunction of dopamine transport and associated functional impairments in subjects with Parkinson's disease (PD), Alzheimer's disease (AD) and dementia with Lewy bodies (O'Brien *et al.*, 2004). Though, the striatonigral system is preferentially affected in PD, extensive neurotoxicity and degeneration is also seen in striatum, hippocampus, and neocortex in cases with cognitive impairment associated with PD (Harding *et al.*, 2002; McKeith *et al.*, 2004). PQ has also been reported to elicit neurobehavioral syndrome (Brooks *et al.*, 1999). PQ has been shown to cross the blood brain barrier (BBB) and is suggested to enter brain through a neutral amino acid carrier due to its structural homology to amino acids (McCormack and Di Monte, 2003; Shimizu *et al.*, 2001; Widdowson *et al.*, 1996). Long-term studies demonstrated that oral ingestion of PQ resulted in brain accumulation of PQ with a much longer half-life (Prasad *et al.*, 2007). ROS generation and the resultant oxidative stress are suggested as one of the primary mechanisms for PQ-induced selective neuronal degeneration. Typically, following cellular exposure of PQ, a cascade of oxidative events are elicited leading to NADPH utilization generating reactive oxygen species (ROS) mainly harmful H₂O₂ and hydroxyl radical (HO.) resulting in lipid peroxidation (Burk *et al.*, 1980).

In general, ROS generated in response to any endogenous or exogenous stimuli is counterbalanced by redox-active cellular factors including enzymatic as well as non-enzymatic antioxidants. Nuclear factor erythroid-2 related factor 2 (Nrf2), a redox-sensitive transcription factor is well documented to tune the expression of array of antioxidant response element (ARE) containing cytoprotective genes and control the physiological and pathophysiological perturbations of oxidants (Yates and Kensler, 2007). Under basal state, Nrf2 is retained in cytoplasm and is maintained at a low level by proteasomal degradation via Keap-1-Cul3/Rbx1/E3-dependent mechanism (Kobayashi *et al.*, 2004). During oxidative stress, the cysteine residue in Keap1 is oxidized and the subsequent conformational change untethers Nrf2 from its latch. Following this event, Nrf2 gains nuclear entry and binds to ARE sequence containing target genes, transactivating them (Nguyen *et al.*, 2000).

However, this adaptive response of Nrf2 is only likely in mild to moderate oxidative stress which is evident from dampened Nrf2 activity and low expression of antioxidant genes in several prolonged/chronic oxidative stress conditions (Gounder *et al.*, 2012; Ramsey *et al.*, 2007). Dysregulation of Nrf2 expression and/or disrupted Nrf2 signaling is suggested to play a critical role in several neurodegenerative conditions including AD and PD (Ramsey *et al.*, 2007). Genetic evidence indicates a link between a complete haplotype (SNPs) of Nrf2 and Keap1 genes in relation to the risk of PD (von Otter *et al.*, 2010). In addition, in severe neurodegenerative conditions, the nuclear trafficking of Nrf2 has been shown to be impaired (Ramsey *et al.*, 2007).

Relevant to the current study, it is important to note that deficiency of Nrf2 and/or its activity could aggravate the Parkinsonism like phenotype and the sustained ROS generated by PQ causes similar patterns of selective vulnerability of dopaminergic neurons as that seen in PD (Betarbet *et al.*, 2000; Brooks *et al.*, 1999; Henchcliffe and Beal, 2008). Keap1 based control of Nrf2 is well studied in various experimental scenarios while the post-transcriptional regulation of Nrf2 which occurs chiefly via microRNA's (miRs) is poorly studied in a given context especially in neurons. Recently, we reported that enforced expression of miRs: 144, 153, 27a and 142-5p elicited a post-transcriptional repression of Nrf2 and its activity in dopaminergic SH-SY5Y neurons (Narasimhan *et al.*, 2012). Thus, the goal of this study was to determine whether PQ exposure would dampen Nrf2 based cytoprotection by a miR-related mechanism in human dopaminergic neuronal SH-SY5Y cells.

2. Materials and Methods

2.1. Materials

SH-SY5Y neuroblastoma cells (CRL-2266) were obtained from ATCC (Manassas, VA). Eagle's minimum essential medium (MEM), antibiotic/antimycotic solution, Trizol were from Invitrogen (Carlsbad, CA). Nutrient mixture F-12 Ham was obtained from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum was from Atlanta biologicals (Lawrenceville, GA) and plasmocin from Invivogen (San Diego, CA). Scramble control anti-miR, anti-miR153 and siPort™ Amine NeoFX were purchased from Ambion (Austin, TX). FuGENE HD was from Roche Diagnostics (Indianapolis, IN). QuantiTect reverse transcription kit for first-strand synthesis and endo free plasmid maxi kit was purchased from QIAGEN (Valencia, CA). Antibodies for Nrf2, GCLC, tubulin, GAPDH, lamin b1 and HRP-conjugated goat secondary antibody were from SantaCruz Biotechnologies Inc. (Santa Cruz, CA). HRP conjugated rabbit secondary antibody was bought from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Caspase 3/7 glo assay and Dual Luciferase assay kit was obtained from Promega (Madison, WI). H₂O₂ and Supersignal West Pico Chemiluminescence detection system was purchased from Thermo Fisher Scientific Inc., (Rockford, IL). 4× ARE Luc plasmid from Dr. Roland C. Wolf (Dundee, UK) and Nrf2 3' UTR WT and Nrf2 devoid of its endogenous full-length 3' UTR region (Nrf2^(-3' UTR)) constructs were kind gift from Dr. Jen-Tsan Chi (Durham, USA). Adenovirus encoding cytoplasmic catalase (Ad cCAT) was a kind gift from Dr. Arthur Cederbaum (New York, USA). The recombinant adenovirus vector containing CMV promoter β-galactosidase (Ad-

CMV- β -gal) was obtained from Vector Biolabs (Philadelphia, PA). Paraquat and all other reagents were from Sigma-Aldrich (St. Louis, MO).

2.2. Cell culture

SH-SY5Y cells were grown in minimum essential medium (MEM)/F-12 HAM nutrient medium with 10% FBS, antibiotic/antimycotic and plasmocin at 37°C in a humidified incubator containing 5% CO₂. All experiments were performed at cell confluency of 70% to 80% between passages 26–33.

2.3. Dose justification of stressors used

SH-SY5Y cells were previously demonstrated to generate more ROS at a PQ concentration ranging from 50 μ M–400 μ M in the absence of marked cell death (Alvarez-Erviti *et al.*, 2013; Gabbianelli *et al.*, 1999; Maracchioni *et al.*, 2007). Based on these reports, a sub-toxic concentration of 50 μ M PQ was used in the present study. During aerobic cerebral metabolism, H₂O₂ is continuously generated reaching up to 100 μ M under certain prooxidative conditions (Hyslop *et al.*, 1995). Importantly, this is the concentration that showed impaired Nrf2 activity (Rojo *et al.*, 2008).

2.4. Transfection and Luciferase assay

Transfection experiments were carried out as previously described (Narasimhan *et al.*, 2011; Narasimhan *et al.*, 2012). The concentrations of different constructs and anti-miRs used per well were as follows: (i) 200 ng each of 4 $\times$ ARE reporter, and 3' WT UTR and mutant reporter (ii) 100 nM of scramble control anti-miR or anti-miR153 (iii) 1 μ g of either pcDNA3 or pcDNA3-Nrf2 without 3' UTR and (iv) 3 ng of renilla reporter used to control for transfection efficiency. Cells were transfected using either siPort amine or Fugene HD. Briefly, miRs (or) vector constructs along with renilla vector and respective transfection reagents were appropriately diluted in OPTI-MEM I medium separately and incubated for 5 min. The plasmid solution is mixed with transfection solution and incubated at room temperature for 20 min allowing the formation of transfection complex. Transfection was performed in serum free, antibiotic free media and 1.5 h post-transfection media containing serum, antibiotics was added and the plates were returned to incubator. After 24 h, cells were treated with indicated concentrations of PQ and H₂O₂ for additional 24 h. At the end of the experiments, gene reporter activity was determined using dual-luciferase reporter assay system according to the manufacturer's instructions (Promega, Madison, WI). The luciferase activity was measured using Glomax 20/20 luminometer (Promega, Madison, WI) and the luciferase activity was normalized to renilla activity or protein concentration obtained from the corresponding samples.

2.5. Adenovirus infection

Cells seeded in six-well plates at 75% confluency were infected with either Ad CMV- β -gal or Ad cCAT. Virus infection was performed in 1 ml of serum free media at a multiplicity of infection (MOI) of 15. After infection, the cells were incubated in the culture hood at room temperature for 1 h following which 1 ml of media containing 20% serum was added to obtain a final concentration of 10 % FBS. The infected plates were then returned to a 37°C

humidified 5% CO₂ incubator. Cells were harvested after 24 and 48 h for initial Western analysis of catalase expression. All experiments involving PQ and H₂O₂ were performed at 24 h post-infection (Narasimhan *et al.*, 2011).

2.6. Immunoblotting

After treatment, cells were washed with cold PBS and lysed in ice-cold RIPA lysis buffer containing protease inhibitor cocktail. The lysates were sonicated and cleared of debris by centrifugation. Equal amounts of protein lysates from various treated groups were separated on 8% polyacrylamide gels using SDS-PAGE, and transferred to a polyvinylidene difluoride membrane, and blocked with 5% nonfat dry milk at room temperature. After washing once with TBS containing 0.1% Tween 20, the membrane were hybridized with primary antibodies against rat Nrf2, GCLC, GAPDH, tubulin and lamin b1. The extensively washed blots were then incubated with horseradish peroxidase-conjugated anti-rabbit or mouse IgG or goat IgG for 1 h. The antigen-antibody complex was detected using an ECL chemiluminescence kit. The immunoreactive bands were quantified by scanning densitometry using ImageJ software and were normalized to the signal intensity of either GAPDH or tubulin.

2.7. Subcellular fractionation

Cells grown in 100 mm petri dish were treated with H₂O₂ or PQ and at the end of experiment, cytosolic and nuclear protein fractions were extracted from cell lysates using NE-PER Nuclear and Cytoplasmic fractionation kit (Thermo Scientific Inc., Rockford, IL), following the manufacturer's instructions. Equal amounts of protein were electrophoresed and immunoblotted for Nrf2 as described above. The quality of the isolation of cytosolic and nuclear fractions was confirmed by immunoblotting analysis using antibody probes specific for GAPDH and lamin b1 respectively.

2.8. RNA isolation and Semi-quantitative real time PCR analysis

Total cellular RNA was extracted using Trizol reagent. 1.5 µg of RNA was incubated with gDNA wipe out buffer (Qiagen, Valencia, CA) at 42°C for 2 min to remove any genomic DNA contamination. Following gDNA elimination, the RNA is reverse transcribed to cDNA using Quantitect reverse transcription kit according to instructions (Qiagen).

For real time PCR analysis of Nrf2, NQO1, GCLC, GAPDH mRNA expression, 1/10th of cDNAs prepared as above was used. Taqman gene expression assays consisting of predesigned primer, probe sets specific for the genes tested and the TaqMan universal mastermix were from Applied Biosystems (Bedford, MA). Real time PCR amplification was performed in 386-well optical plates in a final volume of 20 µL containing 10 µL of TaqMan universal mastermix, 20 pmol of respective primers and 1/10th of reverse transcribed RNA. Real time PCR was conducted on a Biorad CFX384 Real time system (Hercules, CA). The thermal cycling conditions used consisted of 50° C/2 min; 95° C/10 min followed by 40 PCR cycles at 95° C/15 sec and 60° C/1 min. The mRNA expression levels of genes of interest were calculated on the basis of cycle threshold (Ct) value using GAPDH as normalization control. Relative amplification of each transcript was further calculated as fold difference from untreated samples using 2^{-C_t} method (Livak and Schmittgen, 2001).

miRNA detection by real time analysis involved reverse transcription of cDNA using a small RNA specific stem-loop RT primer (hsa-miR153: RT-000476; U6 snRNA: RT-001973). Once specific cDNA was generated, individual miRNA was detected using Taqman small RNA assay real time PCR analysis (hsa-miR153: TM-000476; U6 snRNA: TM-001973). Results were normalized to small nuclear RNA U6 that served as control and the data was expressed as Log 2 fold change in respective miR153/U6 snRNA levels.

2.9. ROS detection by DCF fluorescent assay

Cells were treated with indicated concentrations of the stressors and at the end of the experiments, gently washed once with PBS and then incubated at 37°C for 30 min in dark conditions with 25 µM of cell-permeant, fluorogenic probe, 2', 7'-dichlorofluorescein diacetate (DCF-DA) in PBS. Cells were then gently washed with PBS and scraped with PBS containing 2 mM EDTA in a 96-well clear bottom-white walled plate. Reactive oxygen species oxidize this non-fluorescent DCF-DA to brightly green fluorescent compound 2', 7'-dichlorofluorescein whose fluorescence intensity was measured on GLOMAX Multi Detection System with Optical Kit Blue (Excitation 490 nm and Emission 510–570).

2.10. Caspase 3/7 glo assay

The combined activity of caspases-3/7 was estimated using Caspase-glo 3/7 assay. Briefly, after treatment, cells were washed with PBS and 300 µL of caspase-glo 3/7 reagent was added to each well and cells were scraped, collected in a microfuge tube in dark. The cell lysate was incubated in dark for 30 min and luminescence was read in a Glomax luminometer (Promega). RLU was recorded and results were expressed as fold change in caspase 3/7 activity from control.

2.11. MTT assay

Cell viability was assessed using MTT assay. After incubation of indicated concentration of the stressors for appropriate time, cells were gently washed with phenol red free RPMI-1640 media. MTT was then added at a final concentration of 0.5 mg/ml and incubated at 37° C for 2 h. The purple colored insoluble formazon crystals generated by viable cells were dissolved using an equal volume of solubilizing solution (isopropanol/HCl/Triton X-100). The optical density was recorded at 570 nm in Model 680 microplate reader (BioRad, Hercules, CA). The resultant data was expressed as the percentage of viable cells relative to that of untreated controls.

2.12. Statistical analysis

Data obtained from the experiments were analyzed using Graph Pad Prism software (San Diego, CA). Results are presented as means ± s.e.m. Statistical significance for multiple-group comparisons was assessed using one-way ANOVA followed by a post hoc (Newman-Keuls) test. Two-tailed, paired Student's t-test was used to determine the significance of differences between treated samples and controls. A probability value less than 0.05 was regarded to be statistically significant.

3. Results

3.1. Paraquat upregulates brain enriched, miR153 expression and affect Nrf2 and its transactivation function

Prior research from our laboratory has demonstrated that forced expression of group of miRs viz. miR144, miR153, miR27a and miR142-5p impairs basal Nrf2 and its dependent redox control in SH-SY5Y neurons (Narasimhan *et al.*, 2012). Endogeneous level of miR144 and miR142-5p in this cell is inherently low and was undetectable (Narasimhan *et al.*, 2012). Of note, miR153 is reported to be a brain-specific miRNA and is prominently expressed in neurons (Doxakis, 2010; Sempere *et al.*, 2004). Importantly, a very recent study demonstrated that miR153 overexpression altered neuronal morphogenesis and neuronal development (Wei *et al.*, 2013). On the basis of this premise and PQ being strongly implicated as dopaminergic toxin, we focused herein to test whether PQ influence the expression of brain-enriched, miR153 and target Nrf2 dependent antioxidant function in SH-SY5Y cells. Real time PCR analysis for miR153 was performed on the lysates obtained from untreated, PQ treated cells. miR153 expression, normalized to U6 levels, revealed that PQ significantly ($p<0.05$) induced miR153 expression by $\sim 60\%$ (Fig. 1A). We next examined whether the increase in miR153 might have resulted in suppression of its endogenous target, Nrf2. As expected, real time PCR analysis showed that the miR153 expression was associated with significant ($p<0.05$) downregulation of Nrf2 message normalized to GAPDH levels (Fig. 1B). Nuclear levels of Nrf2 are critical that determines the functionality of Nrf2 and therefore, we next assessed as to whether PQ-induced miR153/Nrf2 message alterations reflected in decline in Nrf2 protein levels in the nuclear compartment. Immunoblotting of Nrf2 using the antibody (sc-722) resulted in multiple bands. Prior studies suggested that Nrf2 anomalously migrates at ~ 100 kDA in SDS-PAGE as against its actual predicted molecular weight 68 kDA due to its inherent high acidic charges (Moi *et al.*, 1994). Other studies report that the ~ 100 kDA form of Nrf2 is ubiquitinated (Li *et al.*, 2005) or is an actin-Nrf2 dimer (Kang *et al.*, 2002). Thus, in our Western analysis, we quantified both ~ 100 kDA and 68 kDA Nrf2 specific bands in the nuclear fractions and were normalized to the levels of lamin-b1. It was observed that ~ 100 kDA and 68 kDA Nrf2-specific bands were significantly decreased in PQ exposed cells (Fig. 1C). Such a regulation of both ~ 100 kDA and 68 kDA for Nrf2 in a given context was reported by us and others (Narasimhan *et al.*, 2012; Rushworth *et al.*, 2011). Next we addressed if the downregulation of Nrf2 is accompanied by its impairment in transactivation function by using two measures: (i) 4 $\times$ ARE reporter assay in which the luciferase construct has 4 $\times$ Nrf2 recognition sites (ii) semi-quantitative real time PCR analysis of Nrf2 target genes, GCLC and NQO1. As measured by luciferase assay, the 4 $\times$ ARE transcriptional activity was significantly decreased approximately ~ 1.6 fold in PQ treated cells when compared to untreated controls (Fig. 1D). In parallel with luciferase transcription assay, the expression of ARE-bearing genes such as NQO1 and GCLC (transcriptional target of Nrf2) was also significantly ($p<0.05$) downregulated in PQ exposed cells (Fig. 1E). The reduction in Nrf2 and its target gene expression triggered by PQ at the tested dosage were significant but the decrease was only about $\sim 20\%$ which does not correlate to the increase in miR153 (60%) (Fig. 1A vs Fig. 1B; Fig. 1D). Such a fine regulation is typical with the miR:mRNA interaction wherein miRs can greatly impact biological outcome with subtle changes on

target genes (Mukherji *et al.*, 2011; Chatterjee *et al.*, 2013; van Jaarsveld *et al.*, 2013). This could be a result of distribution of miR153 among its gene targets as a single miR is known to target several genes (Doxakis, 2010; Broadie and Patton, 2013). Together, these results indicated that PQ induced miR153 is associated with dysregulation of Nrf2 levels and its transactivation function.

3.2. H₂O₂/PQ-induced miR153 resulted in direct targeting of Nrf2 in SH-SY5Y neurons

Typically most forms of cellular oxidative stress lead to copious generation of endogenous H₂O₂, the most stable and highly reactive ROS candidate. As PQ generates ROS (Burk *et al.*, 1980) and H₂O₂ being a pivotal messenger in a given stress-response signaling events, we next tested if H₂O₂ can induce a similar response as that of PQ with reference to miR153/Nrf2 changes. SH-SY5Y cells were treated with H₂O₂ for 24 h and real time PCR analysis demonstrated that H₂O₂ treatment elicited a significant ($p < 0.05$) level of miR153 expression (Fig. 2A). Generally, miRs bind to a ~7 to 8 nucleotide match sequences located in the 3' untranslated region (3' UTR) of their target mRNA and control their expression (Fabian *et al.*, 2010). In order to experimentally determine whether H₂O₂ induced miR153 directly targets Nrf2 by binding to its 3' UTR sequence, we employed reporter assays. Two different reporter constructs were used in this assay: (i) an artificial WT Nrf2 3' UTR construct that harbors intact miR153 site and (ii) miR153 mutated Nrf2 3' UTR luciferase constructs in which the miR153 binding site is three-point mutated (Narasimhan *et al.*, 2012). Both the constructs were individually introduced into the cells following which exposed to H₂O₂. In the wild type transfected conditions, H₂O₂ decreased the luciferase output significantly ($p < 0.05$) by ~35% as H₂O₂-induced miR153 can bind to its intact cognate site. In contrast, the suppressive effect induced by H₂O₂ treatment was lost in mutant miR153 reporter transfected cells (lane 2 vs lane 4; Fig. 2B), suggesting that miR153 directly target these sequences and could suppress gene expression. Next, we verified whether H₂O₂ induced targeting of Nrf2 3' UTR was because of miR153 amplification using anti-miR153 strategy. Cells were transfected with WT Nrf2 3' UTR construct along with antisense-miR153 inhibitor and the effect of H₂O₂ was tested. A non-targeting inhibitor miR (scramble) was used as negative control. Scramble antisense-miR treated with H₂O₂ exhibited relatively low basal luciferase activity (lane 1; Fig. 2C). While, Nrf2 UTR activity was found to be increased in anti-miR153 transfected cells treated with H₂O₂ (lane 2 vs lane 1; Fig. 2C). Further, the results for PQ treatment following similar experimental strategies using WT and mutant Nrf2 3' UTR (Fig. 2D) and anti-miR153 + WT Nrf2 3' UTR (Fig. 2E) were akin to H₂O₂ treatment. This indicates that repression of Nrf2 3'UTR activity by PQ/H₂O₂ could occur due to specific increase in activity of miR153. Overall, these results suggest the following: (i) in order for PQ/H₂O₂-induced miR153 to target Nrf2, the respective miR binding site in Nrf2 3' UTR must be intact. In other words, PQ/H₂O₂ induced miR153 can bind to and directly target Nrf2 3' UTR and (ii) functional silencing of miR153 using specific anti-miR could relieve PQ/H₂O₂-induced repression of Nrf2 3' UTR activity.

3.3. miR153/Nrf2/ARE pathway alterations in response to H₂O₂ are coupled with cell death

Having shown that H₂O₂-induced miR153 directly targeted Nrf2 3' UTR, we next investigated whether this is associated with impairment in Nrf2 levels and its function. To

test this we examined the expression of Nrf2 message using real time PCR and Fig. 3A shows that Nrf2 mRNA normalized to GAPDH expression was significantly downregulated approximately ~2 fold in H₂O₂-treated samples as compared with controls. Since cytosolic and nuclear levels of Nrf2 are critical that determines the functionality of Nrf2, we next assessed as to whether H₂O₂-induced miR153/Nrf2 message alterations reflected in decline in Nrf2 protein levels in this sub-cellular compartments. Immunoblotting of Nrf2 using the antibody (sc-722) demonstrated significant decrease in Nrf2 in both the compartments of H₂O₂ exposed cells (Fig. 3B & 3C). Next, we assessed if the decreased nuclear Nrf2 in response to H₂O₂ was associated with impaired 4× ARE transcriptional regulation. Fig. 3D illustrates that H₂O₂ effected a significant ($p<0.05$) downregulation of Nrf2-dependent transcription which is evident from ~30% decrease in 4× ARE luciferase activity over basal. Reporter gene overexpression based transcriptional activity measures does not represent the true endogenous setting and thus we verified Nrf2's transcriptional activity by measuring endogenous expression of GCLC, an Nrf2 target. In parallel with 4× ARE luciferase estimates, GCLC protein content was found to be significantly decreased in H₂O₂ treated cells (~40% decrease; $p<0.05$; Fig. 3E). Since imbalance of cellular antioxidant pathway in response to H₂O₂ has been shown to affect cell survival (Zhang *et al.*, 2007), we next assessed cell death using MTT assay. In accordance with several findings, our results demonstrate that the cell viability was significantly reduced by ~30% (Fig. 3F). Altogether these results along with Fig. 2 demonstrate that H₂O₂ impairs Nrf2/ARE defense network through the induction of miR153 and all of these events are associated with cell death of these dopaminergic neurons.

3.4. miR153 mediated blunting of Nrf2-directed ARE activity influences cell death

To further investigate whether PQ/H₂O₂ induced miR153 mediated suppression of 4× ARE transactivation activity occurs through Nrf2 downregulation, we performed co-transfection experiments. In this assay, cells were transfected with plasmids encoding Nrf2 devoid of its endogenous full-length 3' UTR region (Nrf2^(-3' UTR); not containing miR sites) facilitating a miR153 non-targetable Nrf2 mRNA. pcDNA3 empty vector (without Nrf2 cDNA sequence) was used as control. These constructs were individually introduced into cells along with 4× ARE reporter construct. 24 h post-transfection, cells were exposed to either PQ or H₂O₂. We observed significant ($p<0.05$) suppression in 4× ARE transcriptional activity in PQ or H₂O₂-treated control vector transfected cells (lane 2 vs lane 1; Fig. 4A, Fig. 4B respectively) indicating a PQ/H₂O₂ augmented miR153 and its accessibility for Nrf2 3' UTR that in turn, can lower Nrf2 and its activity. In contrast, a significant ($p<0.05$) inverse correlation between PQ/H₂O₂-induced reduction of luciferase activity of 4× ARE construct with the overexpression of Nrf2^(-3' UTR) was observed (lane 4 vs lane 2; Fig. 4A & Fig. 4B). In this case, though PQ/H₂O₂ induced miR153 can bind to and target endogenous Nrf2 3' UTR, since the cells were primed with Nrf2 lacking 3' UTR, PQ/H₂O₂ elicited reduction in 4× ARE activity were rescued. This result supports the notion that altered expression of Nrf2 by miR153 is indeed responsible for PQ/H₂O₂ evoked repression of ARE activity. Having shown this, we next investigated whether blocking miR153 prevents the loss of PQ/H₂O₂ induced 4× ARE activity. To study this, either scramble anti-miR or anti-miR153 inhibitor along with 4× ARE reporter construct was introduced into SH-SY5Y cells. 24 h post-transfection, PQ or H₂O₂ treatment was performed for additional 24 h and the lysates were

assessed for luciferase activity. A ~2 fold decrease ($p < 0.05$) in 4× ARE transcriptional activity was noted in scramble transfected cells (lane 2 vs lane 1; Fig. 4C, Fig. 4D respectively). On the other hand, overexpression of anti-miR specific against miR153 drastically antagonized the PQ/H₂O₂-induced reduction in 4× ARE transcriptional activity ($p < 0.05$) (compare fold difference lane 1, 2 vs lane 3, 4; Fig. 4C & Fig. 4D). We next verified the endogenous expression of GCLC (one of the ARE promoted antioxidants), since luciferase assay uses exogenous expression of ARE containing elements thus not representing the actual cellular ARE driven gene expression. Similar anti-miR strategy with and without H₂O₂ treatment (as in Fig. 4D) was carried out followed by immunoblotting for GCLC. Analogous to luciferase assays, H₂O₂ induced diminution of cellular GCLC levels observed in scramble miR transfected cells (lane 1 vs lane 2; Fig. 4E) was abolished in anti-miR153 overexpressed cells (lane 2 vs 4; Fig. 4E). Thus, data from Fig. 4A-4E denotes that PQ/H₂O₂ prompted suppression of ARE-driven gene expression could be restored by inhibiting miR153 in SH-SY5Y dopaminergic neurons. Since H₂O₂ induces death of dopaminergic neurons and maintenance of redox balance affords cellular protection (Zhang *et al.*, 2007), we next sought to determine whether Nrf2^(-3' UTR) that lacks miR153 binding site may confer protection against H₂O₂. Empty vector transfected cells displayed ~30% ($p < 0.05$) cell death in response to H₂O₂, while, Nrf2^(-3' UTR) overexpression normalized H₂O₂ induced cell death (Fig. 4F). Overall these results illustrate that (i) PQ/H₂O₂ induced miR153 hinders ARE activity via impairing Nrf2 and this can be reversed by inhibiting miR153 (ii) H₂O₂ induced miR153-mediated Nrf2 repression could play a key role in oxidant stress related dopaminergic neuronal death.

3.5. Paraquat- induced deregulation of miR153/Nrf2/ARE network is ROS (H₂O₂)-dependent

Since we have shown that PQ and exogenous H₂O₂ treatment induce miR153 and affect Nrf2/ARE pathway (Fig. 1-4), we next evaluated the role for intracellular H₂O₂ in PQ elicited damages to miR153/Nrf2/ARE pathway. Typically, intracellular level of H₂O₂ is delicately balanced by action of several antioxidant proteins including catalase (Rhee *et al.*, 2000). Thus to test this we performed overexpression experiments with adenovirus encoding cytoplasmic catalase (Ad cCAT). The recombinant adenovirus vector containing CMV promoter β-galactosidase (Ad-CMV-β-gal) was used as control. The efficiency of catalase virus was first verified by immunoblotting in SH-SY5Y cells. Fig. 5A demonstrates that increase in catalase expression was noted as early as 24 h post-infection of Ad cCAT when compared with Ad-CMV-β-gal infection. This increase in catalase level was found to be sustained till 48 h (Fig. 5A). Given that PQ triggers H₂O₂ production and catalase serving as the major scavengers of H₂O₂, it follows that overexpression of catalase could abolish PQ-induced amplification of miR153. To evaluate this, we infected the cells with either Ad-CMV-β-gal or Ad cCAT 24 h prior to PQ treatment and analyzed the expression of miR153. As expected, the real time PCR analysis demonstrated significant suppression of PQ-induced miR153 by ectopic expression of catalase ($p < 0.05$) (lane 4 vs lane 2; Fig. 5B). These cells exhibit low GPx activity (Filomeni *et al.*, 2012) and thus expected to have higher intrinsic H₂O₂ levels regulating the basal miR153 expression. So, catalase expression would hypothetically decrease the basal miR153. In view of this anticipation, we observed a moderate but not significant decrease in basal miR153 levels on Ad cCAT overexpression (lane 3 vs lane 1; Fig. 5B) indicating a partial role for H₂O₂ in basal regulation of miR153.

We next validated whether or not the catalase mediated inhibition of PQ-induced miR153 results in recovery of its target gene, Nrf2. In association with blocking miR153 levels, catalase overexpression significantly limited the PQ induced decline in Nrf2 mRNA level ($p<0.05$) (lane 4 vs lane 2; Fig. 5C). Though the miR153 induction response to PQ is higher in adenovirus based experiments when compared to uninfected experimental set up (~ 3 fold in Fig. 5B vs ~ 1.9 fold Fig. 1A), the magnitude of Nrf2 repression was almost identical. Notably, replication-defective recombinant adenovirus containing no foreign gene (Ad CMV/Ad-CMV- β -gal) has been demonstrated to induce oxidative stress that are marked by the activation of AP-1 binding, c-jun along with decrease in GSH/GSSG ratio, alterations in mitochondrial and host-cell environment (Zhang et al., 2001; Alesci et al., 2002; Hoenig et al., 1974; Margolis et al., 1974). Thus, we speculate that adenovirus transduction could prime (oxidant) basal ROS machinery as well as its dependent transcription factors that could enhance miR153 response to PQ (Fig. 5B). Further, the reason for identical strength of Nrf2 repression among the uninfected and adenovirus experimental set up despite higher miR153 response to PQ may be ascribed to co-induction of a “sponge” phenomenon in mammalian and non-mammalian organisms (Ebert and Sharp, 2010) (yet other reasons detailed in the discussion could also play a role). Subsequently, the involvement of H_2O_2 in PQ-mediated impairment of Nrf2 function was confirmed by assessing the expression of GCLC, an Nrf2 target. It is clear from immunoblotting analysis that PQ induced significant downregulation of GCLC expression in Ad gal transfected control cells ($p<0.05$) (lane 3 vs 1; Fig. 5D). On the contrary, infection of cells with Ad cCAT restored the PQ-induced loss of GCLC levels (compare lane 3 vs lane 4; Fig. 5D). These data suggests that stressors like PQ that stimulates H_2O_2 generation can upregulate miR153 thereby impairing Nrf2 dependent cellular antioxidant capacity. This can be attenuated by catalase overexpression.

3.6. Catalase inhibits PQ-evoked miR153 mediated redox-related cellular alterations

Above data indicate that alterations in Nrf2/ARE pathway is an outcome of increased miR153 during PQ exposure (Fig. 5). Since we and others have reported that dysregulation of the Nrf2/ARE network enhances intracellular ROS (Aw Yeang *et al.*, 2012; Harvey *et al.*, 2009; Narasimhan *et al.*, 2011; Narasimhan *et al.*, 2012), we next sought to evaluate whether the Nrf2/ARE changes induced by PQ elevate intracellular ROS levels. In agreement, the DCF based fluorescence assay revealed that PQ significantly ($p<0.05$) induced endogenous ROS levels by $\sim 25\%$ (lane 2 vs lane 1; Fig. 6A). However, in cells overexpressing catalase, PQ failed to invoke ROS production (lane 2 vs lane 4; Fig. 6A) suggesting that the cellular redox equilibrium could be shielded by neutralizing H_2O_2 and its associated disturbances in miR153/Nrf2/ARE cascades. To strengthen our hypothesis that ROS induced cell death precedes activation of effector caspases namely caspase 3 and caspase 7, we measured the activity of caspase 3/7 by glo assay. Fig. 6B shows that PQ-induced caspase 3/7 activity (lane 2 vs lane 1) was significantly ($p<0.05$) minimized by catalase overexpression (lane 2 vs lane 4; Fig. 6B). To ascertain that these events follow cell death, we utilized the MTT assay. It is evident that Ad cCAT infected SH-SY5Y neurons underwent minimal apoptosis following PQ treatment (lane 2 vs lane 4; Fig. 6C) as compared to Ad-CMV- β -gal infected cells (lane 2 vs lane 1; Fig. 6C). Taken together, these results suggest that if PQ-triggered H_2O_2 is inactivated, the subsequent H_2O_2 -dependent miR153 mediated impairment in Nrf2/ARE signaling cascade could be corrected thus conferring neuroprotection.

4. Discussion

Despite extensive progress in the field of Nrf2-redox biology, mechanisms underlying how persistent and/or prolonged oxidative stress dampens Nrf2 cytoprotective responses enabling cells to undergo death are not fully elucidated. In this study, we report for the first time that miR153 suppresses Nrf2/ARE cascade and its associated cytoprotection during PQ-induced toxicity in dopaminergic neurons. The major findings of this study are: (i) miR153 is significantly upregulated in response to a physiologically relevant level of PQ (ii) This miR153 increase elicits inactivation of Nrf2 and its antioxidant functions (iii) Silencing of miR153 by anti-miR153 rescued PQ and H₂O₂-induced Nrf2 3' UTR targeting and Nrf2's transcriptional activity (iv) PQ-elicited miR153 is H₂O₂ dependent (v) Catalase-mediated reduction of cellular H₂O₂ prevented PQ-related induction of miR153 thereby safeguarding Nrf2/ARE cytoprotective machinery and reducing oxidative cellular damage. Thus, minimizing miR153 levels could be a critical factor in governing cellular integrity during PQ-induced neurotoxicity. The overall findings of the current study are summarized in a schematic (Fig. 7).

A plethora of studies have documented that Nrf2 protects multiple organs against diverse toxic insults, functioning as a multi-organ protector (Lee *et al.*, 2005). We and many others have shown that Nrf2 is activated during oxidative stress in a variety of settings (Harvey *et al.*, 2009; Kang *et al.*, 2002; Li *et al.*, 2005; Narasimhan *et al.*, 2011). However, it is evident that such Nrf2 activation is a typical stress response that can occur only during mild to moderate oxidative stress. This was recently supported in a classical non-neuronal cell culture based study wherein short-term exposure of astrocyte-rich cultures to a ROS-generating cytokine, TNF α was shown to upregulate Nrf2. However, this increase reversed following 72 h treatment of TNF α (Correa *et al.*, 2012). In addition, the Nrf2 system can be dysfunctional in severe and/or chronic oxidative neurodegenerative pathologies such as PD, AD and Lewy body variant of AD (LBVAD) (Ramsey *et al.*, 2007). So far, mechanisms like nuclear trafficking involving GSK-3 β signaling changes or Keap1 are suggested to inactivate Nrf2 during persisting oxidant milieu or post-induction repression of antioxidant response (Rojo *et al.*, 2008; Sun *et al.*, 2007). Notably, each cellular system is unique and several pathways could intersect in controlling a pivotal molecule like Nrf2 in a given context. Equally important is that miR mediated post-transcriptional control of a gene product occurs relatively rapid when compared to transcriptional or translational based regulatory mechanisms.

In line with such views, we identified miR153 as a key regulator that is induced during paraquat exposure and it disrupts Nrf2-redox control in dopaminergic cells. Though we observed a significant reduction in the levels of Nrf2 in response to PQ, the decrease was only about ~20% which was not comparable to the percentage increase in miR153 (Fig. 1A; Fig. 1B). Recently it has been demonstrated that the absolute expression level of miRNA need not necessarily predict its inhibitory potential as the availability of repertoire of mRNA targets level could titrate away the miRNAs and thus its association with RISC complex (De *et al.*, 2013; Flores *et al.*, 2014) hence, saturating the level of RISC loading of a cognate miRNA in a given stress context (herein, PQ). Further, PQ could likely co-induce the expression of miR153 and its specific competing endogenous “miRNA sponges” (ceRNAs)

[such as endogenously transcribed pseudogenes, long noncoding RNAs, and recently discovered circular RNAs that could sequester a fraction of a particular miRNA]. This could likely fix a high threshold above which miR153 must rise to effectively repress the expression of a critical cytoprotective gene like Nrf2. In general, stress-induced sponges could act as a quality control/buffer mechanism and ensure that the critical genes fundamental to cell survival are not readily targeted (Bak and Mikkelsen, 2014; Poliseno et al., 2010; Cesana et al., 2011) instead undergo a programmed damage averting a sudden collapse by knocking off Nrf2 hugely. In other words, this is to maintain a gene expression thresholding, one of the key features of cell-fate decisions thus providing an recovering opportunity in case of waning off the toxin, PQ. This is also evident from our earlier report, wherein ectopic expression of miR153 resulted in log (2) 8.5 fold in levels of miR153 with only ~25% of Nrf2 targeting in SH-SY5Y cells (Narasimhan et al., 2012). In such situations where changes in miRNA levels may not be exactly reflected in the target gene regulation, it is recommended that a miRNA-gene regulation be supported by evaluating protein levels and/or any functional output of the gene. To this end, we have supported our gene expression data with protein level (Nrf2) (Fig. 1C), 4× ARE functional assay- (Nrf2 dependent) (Fig. 1D) and gene expression of GCLC, NQO1 (targets of Nrf2) (Fig. 1E), all of which points out an inverse correlation between miR153 to Nrf2 and its associated functions in response to PQ. In addition, this novel finding of miR153 based Nrf2 regulation could be yet another mechanism through which PQ continues to maintain oxidative stress and severe mitochondrial dysfunction (Alvarez-Erviti et al., 2013; Fukushima et al., 1995; Gabbianelli et al., 1999; Maracchioni et al., 2007). Although the focus of the present study addresses miR153-Nrf2 antioxidant machinery, it is likely that other redox and non-redox aspects of cellular changes are impacted by induction of miR153.

In the last few years, miR based mechanisms have been demonstrated in several neurodegenerative diseases e.g. AD, PD, spinocerebellar ataxia type I etc. (Hebert et al., 2008; Kim et al., 2007; Lee et al., 2008). Very recently, enforced expression of miR153 was shown to reduce the expression of Nrf2, bassoon, SNAP25 and SNCA in SH-SY5Y neuronal cells (Mandemakers et al., 2013; Narasimhan et al., 2012). In the current study, we have determined a previously unknown mechanism of PQ-mediated neurotoxicity with respect to increased miR153 and altered Nrf2 system suggesting miR153 targeting could be beneficial. However, a recent report showed that miR153 is essential for proper neuronal patterning and associated functions during early zebrafish development (Wei et al., 2013). This raises an important issue as to the need to identify specific functions of this miR within the context of species and specific cell types. A vital function would be as a regulator during development of neuronal system, ROS based mechanisms are required for appropriate lineage specification of neural progenitors, cellular growth, differentiation, neuronal plasticity (Klann and Thiels, 1999; Le Belle et al., 2011; Prozorovski et al., 2008; Suzukawa et al., 2000; Tsatmali et al., 2006). Further, ROS-induced apoptosis may be a means to selectively remove superfluous neurons during brain development thereby sculpting the size and ultimately, functionality of the nervous system (Dekkers et al., 2013; Oppenheim, 1991). If the above hold true, regulating miR153 as an intervention will need to take into account development dependent balances where a specific manipulation is favorable at one stage and detrimental at the other e.g. "antagonistic pleiotropy" (Williams, 1957). Thus, it is

possible that miR153-Nrf2 fine tuning may fall under this interesting category, as recently found for E74A gene-miR34 (Liu *et al.*, 2012) and hinting at a need for more focused contextual based approach to modulate endogenous miR153 levels. Our results using mutation and anti-miR strategies provide additional insights as to how miR153 can directly target Nrf2 and its related antioxidant system, and emphasize the need for controlling miR153 for dopaminergic neuronal survival during imminent oxidative stress.

Mechanistically, ROS can alter the structure of DNA, protein. H₂O₂, a relatively stable ROS, can induce greater structural perturbation of cellular RNA than DNA (Hofer *et al.*, 2006). However, the means by which ROS regulates non-coding RNAs like miRs in the nervous system is still under investigation. That this occurs for the mature miR153 determined in our studies, was confirmed by H₂O₂ and gain-of-function experiments with catalase overexpression in dopaminergic neurons. In the PQ setting, H₂O₂ was indispensable for miR153 upregulation and associated Nrf2 impairment that is evident from catalase experiments. The mechanism by which PQ-induced ROS/H₂O₂ mediates miR153 expression is unknown at this point. However, we speculate that a ROS responsive transcription factor p53 could either induce direct upregulation as in the case of miR34a (Raver-Shapira *et al.*, 2007) or increase Drosha-mediated post-transcriptional maturation (Suzuki *et al.*, 2009). Studies are underway in our laboratory to investigate as to how ROS/H₂O₂ elicits endogenous miR153.

Previously several studies have sought to determine the effect of PQ on neuronal function and death. Suggested mechanisms for this neurotoxin-induced cell death included energy failure, mitochondrial dysfunction, redox imbalance, autophagy etc., (Alvarez-Erviti *et al.*, 2013; Gabbianelli *et al.*, 1999; Maracchioni *et al.*, 2007; Mollace *et al.*, 2003). Within the context of PQ toxicity, we are not aware of any reports describing how miRNA based fine tuning events can modulate a key Nrf2 dependent nodal regulatory point in cytoprotective signaling in a dopaminergic neuron model. In this connection, the current study is the first to reflect a very important mode of miR153 based Nrf2 dampening response occurring in protracted oxidative stress. Of note is that the current model of PQ addresses only the neurotoxicity aspect (not the late degeneration stage). As ROS is continuously being generated and the cells are in progressive stage of cell death (~35 % cell death; Fig. 6C), *de novo* biogenesis of miR153 is still a possibility that enables the cells to maintain miR153 at a fairly high level. Notably, elevated and prolonged oxidative stress occurs after a single intraperitoneal exposure to PQ, with brain levels ranging between 0.1-0.2 ng PQ/mg tissue (Prasad *et al.*, 2007). Therefore, disrupting miR153 could be a reasonable means to restore Nrf2/ARE signaling-related cytoprotection. Since ROS generation is a trigger for miR153 increase and at the same time a mild/moderate oxidative stress induces Nrf2, we propose an interesting co-regulation of a miR153 and its target during early PQ exposure. In such a case could this be used as a sensor for the upcoming degenerative events? Studies are emerging detailing detection of cellular miRs in plasma, whole blood and leukocytes from PD, AD patients and suggested a non-invasive strategy to diagnose early PD and AD (Cardo *et al.*, 2013; Khoo *et al.*, 2012; Leidinger *et al.*, 2013; Soreq *et al.*, 2013). Figure 7 illustrates the overall schema of miR153 working in sync to regulate the Nrf2/ARE network in PQ neurotoxicity.

Conclusions

Our study suggests that PQ-evoked miR153 targets the Nrf2-antioxidant network resulting in aberrant cellular survival events. Further, changes in miR153 levels may likely predict the progression of PQ-induced redox related neuropathology.

Implications

Dopaminergic neuron systems regulate critical functions including motor control and cognition. Damage to these is linked to a plethora of psychiatric and neurological disorders. Considering the neurotoxic effects of PQ and the emerging roles of miRs in neuro-pathogenic mechanisms, studies of this type will provide a better understanding of the mechanistic intricacies of dopaminergic neuron protective systems and could potentially lead to means of enhancing their neuroprotective responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by RO1 AA010114 (National Institutes of Health Research Project Grant Program) to George I. Henderson. We thank Dr. Jen-Tsan Chi (Durham, USA) for Nrf2 3' UTR WT; Dr. Roland C. Wolf (Dundee, UK) for 4x ARE Luc plasmid; Dr. Arthur Cederbaum (New York, USA) for adenovirus encoding cytoplasmic catalase (Ad cCAT).

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Highlights

- Paraquat and H₂O₂ induces miR153 in SH-SY5Y dopaminergic neuronal model
- Paraquat and H₂O₂ decreased Nrf2 gene expression and associated transactivation function is miR153 dependent
- Catalase-mediated reduction of cellular H₂O₂ prevented PQ-related induction of miR153 and reinstated Nrf2/ARE cytoprotection

Fig. 1. Paraquat induced upregulation of brain enriched miR-153 expression is associated with downregulation of Nrf2 and its transactivation function

SH-SY5Y cells were treated with indicated concentrations of paraquat for 24 h. (A) At the end of the experiment, cDNA was reverse transcribed using a small RNA specific stem-loop RT primer (for miR153 and U6). Semi-quantitative real time PCR analysis was performed using Taqman small RNA assay and results were normalized to small nuclear RNA U6 that served as control. The analyzed data was expressed as Log 2 fold change in miR153/U6 snRNA levels from 4 independent samples. (B) TaqMan based semi-quantitative real time RT-PCR analysis of Nrf2 mRNA level in SH-SY5Y cells treated with indicated concentration of PQ. GAPDH mRNA levels were used to normalize those of Nrf2 mRNA (n=4). (C) Nuclear extracts were immunoblotted for Nrf2, lamin-b1 and GAPDH. The panel below the representative image depicts the quantification of ~100 kDa (represented by *) and 68 kDa (represented by #) specific bands corrected to lamin b1 expression using Image J software (n=3). Low and high exposure was provided to note the changes in ~100 kDa and 68 kDa respectively. (D) Cells were transfected with 200 ng of luciferase construct containing 4x ARE sequences along with 3 ng of pRL-renilla construct. 24 h post-transfection, cells were treated with indicated concentration of PQ for additional 24 h. Nrf2 transactivation was determined in terms of measuring the reporter activity. Percentage change in luciferase activity normalized to renilla was plotted (n=6). (E) Endogenous NQO1

and GCLC mRNA expression from cells treated with and without PQ were estimated by Taqman based semi-quantitative real-time RT-PCR analysis. NQO1 and GCLC mRNA levels were normalized to GAPDH (**n=4**). In panel (A-D), values are expressed as means + s.e.m and two-tailed paired Student's t-test was used to establish the statistical significance. * - $p < 0.05$ compared with untreated control.

Fig. 2. PQ/H₂O₂ induced miR153 targets Nrf2 in SH-SY5Y cells

(A) SH-SY5Y cells were treated with H₂O₂ for 24 h and semi-quantitative real time RT-PCR analysis for miR153 and U6 was performed. Data is expressed as relative intensity of miR153 normalized to U6 over untreated control (**n=4**). (B) SH-SY5Y cells were co-transfected with 200 ng of reporter gene (either wild type or miR 153 site mutated Nrf2 3' UTR) constructs along with 3 ng of pRL-renilla constructs. 24 h following transfection, cells were exposed to 100 μ M H₂O₂. At the end of the treatment, firefly luciferase activity normalized to renilla activity was determined. The results are represented as percentage change over respective controls (**n=6**). (C) Luciferase reporters containing wild-type human Nrf2 3' UTR were co-transfected with scramble control anti-miR or anti-miR-153 into SH-SY5Y cells. 24 h after transfection, cells were treated with 100 μ M H₂O₂ and following which luciferase activity was measured. After normalization for protein levels, the results were plotted (**n=6**). (D) Cells were transfected and processed as in Panel B but for treatment with PQ (**n=4**). (E) Cells were transfected and processed as in Panel C but for treatment with PQ (**n=4**). In (A-E), values are expressed as means + s.e.m. * indicates significant differences at $p < 0.05$ by either one way ANOVA/Newman Keul's post-test or two-tailed paired Student's t-test; ns-not significant.

Fig. 3. Nrf2 is a functional downstream target of miR153 during H₂O₂ induced cell death
 (A) Semi-quantitative RT-PCR analysis of Nrf2 expression in SH-SY5Y cells 24 h post-treatment of 100 μ M H₂O₂. Endogenous Nrf2 mRNA levels were normalized to GAPDH (**n=4**). (B) Cytosolic and (C) nuclear extracts from either untreated or H₂O₂ exposed cells were immunoblotted for Nrf2. Tubulin and lamin b1 served as controls for purity of cytosolic and nuclear fraction respectively. * and # indicate 100 kDA and 68 kDA Nrf2 bands respectively. In panel C, low exposure and high exposure was provided to appreciate the reduction in ~100 kDA and 68 kDA respectively. Using ImageJ based densitometric analysis, the intensity of 100 kDA and 68 kDA Nrf2 levels were normalized to either tubulin or lamin b1 and plotted. A representative blot is given in B & C from 3 independent experiments. (D) 4x ARE luciferase reporter construct transfected cells were treated with or without H₂O₂ for 24 h followed by luciferase measurements. Luciferase activity normalized to protein was plotted and values are the averages of **n=6**. (E) Immunoblotting analysis of GCLC expression levels in SH-SY5Y cells treated with 100 μ M H₂O₂ and a representative image is given (**n=3**). The respective densitometric scanning ratio of GCLC/tubulin is depicted in the lower panel. (F) SH-SY5Y cells were treated with indicated concentration of H₂O₂ and MTT assay was performed as described in materials and methods. The data are represented as percentage of cell viability relative to untreated cells. Each bar represents the

mean $\pm$ s.e.m from 6 independent determinations. In panels (A-F), Student's t-test determined the significance of treatment. * - represents $P < 0.05$ vs untreated controls (mean $\pm$ s.e.m).

Fig. 4. Role for intact Nrf2 3' UTR/miR153 interaction in H₂O₂ induced cell death

(A) 4× ARE luciferase reporter and pRL renilla constructs were co-transfected with either Nrf2 devoid of its endogenous full-length 3' UTR region (Nrf2^(-3' UTR); lacking miR sites) or empty vector. 24 h post-transfection, cells were treated with or without PQ for 24 h. Dual luciferase measurements were performed and the data are expressed as fold change relative to respective control. Luciferase activity normalized to renilla activity was plotted and values are the averages of **n=4**. (B) Cells were transfected and processed for luciferase activity as in Panel A but treated with H₂O₂ for 24 h after transfection (**n=6**). (C) Co-transfection assays involving 4× ARE reporter constructs along with anti-miR153 was performed as indicated in materials and methods. 24 h post-transfection, cells were treated with PQ for additional 24 h. Extracts prepared at the end of the experiment was processed for dual luciferase assay. Relative luciferase units were normalized to renilla activity and the percentage change over respective controls were plotted (**n=4**). (D) Cells were transfected and processed for luciferase activity as in Panel C but underwent 24 h H₂O₂ treatment post-transfection (**n=6**). (E) Scramble control anti-miR or anti-miR153 transfected cells were treated with or without H₂O₂ and immunoblotting for GCLC and GAPDH was performed. Relative fold change in expression of GCLC normalized to GAPDH was indicated. (F) Transfection and treatment without 4× ARE and pRL renilla reporter constructs were

performed as in Fig. 4A. Cell viability was measured using MTT assay and % live cells were plotted (**n=6**). In panels (A, B, C, D, F), values are expressed as mean $\pm$ s.e.m and statistical significance was established using one way ANOVA/Newman Keul's post-test. * - represents $P<0.05$ vs untreated controls and ns indicates not significant.

Fig. 5. Paraquat-induced H₂O₂ induces miR153 and impairs Nrf2/ARE pathway

(A) Cells were infected with 15 MOI of adenovirus encoding cytoplasmic catalase (Ad cCAT) or Ad-CMV- β -gal. 24 h and 48 h later, cell extract was prepared and 25 μ g protein was immunoblotted for catalase and GAPDH. As described in Fig. 5A, cells were infected with the viruses for 24 h and PQ was treated for additional 24 h. At the end of treatment, cells were processed for semi-quantitative real time PCR analysis for miR153 and U6 (B); Nrf2 and GAPDH (C). miR153 and Nrf2 were normalized to U6 and GAPDH levels respectively. (D) Infection and treatment was carried out as in Fig. 5B, and proteins were analyzed by immunoblot with anti-GCLC, anti-catalase and anti-tubulin. Panel below the representative image depicts the quantification of GCLC expression corrected to tubulin using Image J software. In panels (B, C & D), data are expressed as mean $\pm$ s.e.m and one way ANOVA/Newman Keul's post-test was used for statistical analysis. * - represents $P < 0.05$.

Fig. 6. Effect of catalase in preventing paraquat-induced ROS, caspase3/7 and cell death
Infection of catalase virus and paraquat treatment was performed as described in Fig. 5B. At the end of the treatment, cells were processed for intracellular ROS generation by DCF based fluorescence assay (A), caspase 3/7 activity by glo assay (B) and cell viability by MTT assay (C). Data are expressed as mean $\pm$ s.e.m ($n=6$) and one way ANOVA/Newman Keul's post-test was performed to establish statistical significance. * - represents $P<0.05$.

Fig. 7. Schematic showing the role of miR153 in paraquat elicited neurotoxicity
 H₂O₂ generated on paraquat exposure, can evoke the expression of miR153. Increased miR153 could then target Nrf2 mRNA by binding to its 3' UTR resulting in decreased levels of Nrf2 protein. Further, this could impair transcription of Nrf2 dependent antioxidant genes leading to cascade of adverse events such as increased ROS and activation of effector caspases 3/7 affecting the survival of dopaminergic neurons.