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DOWN-REGULATION OF LRRK2 IN CONTROL AND DAT TRANSFECTED HEK CELLS INCREASES MANGANESE-INDUCED OXIDATIVE STRESS AND CELL TOXICITY

Jerome A. Roth and Michelle Eichhorn

Department of Pharmacology and Toxicology, University at Buffalo, Buffalo, NY 14214

Abstract

The extra-pyramidal symptoms associated with manganism often overlap with that seen in Parkinsonism suggesting a common link between the two disorders. Since wide deviations are observed in susceptibility and characteristics of the symptoms observed in manganism, these differences may be due to underlying genetic variability. Genes linked to early onset of Parkinsonism which includes ATP13A2 and parkin have already been suggested to promote development of Mn toxicity. Of the other Parkinson-linked genes, mutations in LRRK2, an autosomal dominant gene, represent another likely candidate involved in the development of manganism. In this paper the effect of shRNA LRRK2 knock-down on Mn toxicity was examined in control and DAT transfected HEK293 cells. Results demonstrate that LRRK2 down-regulation potentiates Mn toxicity in both control and DAT-transfected cell as well as potentiates DA toxicity. Combined treatment of Mn and DA further augments cell toxicity, ROS production and JNK phosphorylation in LRRK2 deficient cells compared to controls. Consistent with studies demonstrating that LRRK2 plays a role in the phosphorylation of p38, our results similarly demonstrate a decrease in p38 activation in LRRK2 knock-down cells. Our findings suggest that null mutations in LRRK2 which cause Parkinsonism potentiate Mn toxicity and increase susceptibility to develop manganism.

Keywords

Manganese; Manganism; Parkinson's disease; Dopamine; HEK293; Dopamine transporter; LRRK2

1. Introduction

Chronic exposure to Mn has been linked to development of a severe neurological disorder known as manganism consisting of reduced response speed, intellectual deficits and compulsive behaviors in the initial stages of the disorder to more prominent and irreversible extrapyramidal dysfunction resembling Parkinson's disease upon protracted exposure (Huang *et al.*, 1993; Krieger *et al.*, 1995; Mena *et al.*, 1969; Mergler and Baldwin, 1997;

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Corresponding author: Jerome A. Roth, Department of Pharmacology and Toxicology, 11 Cary Hall, University at Buffalo, Buffalo, NY 14214, Phone: 716 829 3236, jaroth@buffalo.edu.

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Olanow *et al.*, 1996; Pal *et al.*, 1999; Pomier-Layrargues *et al.*, 1995). Mn neurotoxicity is primarily an occupational disorder associated with environmental conditions in which workers are exposed to chronic high levels of the metal (Krieger *et al.*, 1995; Pomier-Layrargues *et al.*, 1995) and include Mn miners, welders and those involved in ferroalloy and battery production.

Although the identified brain lesions associated with manganism differ, in part, from those of Parkinson's disease, recent studies have revealed that Mn can also disrupt dopaminergic activity in the striatum which may be responsible for some of the observed extra-pyramidal symptoms overlapping with that of Parkinsonism (Guilarte, 2011; Guilarte *et al.*, 2008; Peneder *et al.*, 2011; Sriram *et al.*, 2010). This is consistent with the fact that Mn has been reported to be one of the most influential metals associated with increased susceptibility to develop Parkinson's disease further implying a possible common link between the two disorders (Criswell *et al.*, 2012; Gorell *et al.*, 1999; Racette *et al.*, 2001). Since wide deviations are observed in onset, susceptibility and severity of the symptoms observed in manganism, it is not unreasonable to hypothesize these differences are due to underlying genetic variability which may be linked to inheritable factors associated with early and late onset Parkinsonism. Several genes have recently been identified with provocation of Parkinson's disease including parkin, α -synuclein, DJ-1, PINK, ATP13A2 and LRRK2 (Kumar *et al.*, 2011). Because of the common features connecting the two disorders, the question can be asked as to whether mutations in these genes also correlate with propensity to develop Mn toxicity. In fact, two of these genes, parkin and ATP13A2, have already been reported to protect cells from the neurotoxic actions of Mn (Gitler *et al.*, 2009; Roth, 2009; Roth *et al.*, 2010). Thus, one-third of the known polymorphisms in genes associated with juvenile and/or late onset Parkinsonism have now been acknowledged to potentially correlate with development of manganism raising the question as to whether any of the other genes associated with Parkinsonism are similarly linked to Mn toxicity. This paper specifically focuses on the effects of LRRK2 on Mn toxicity principally because mutations in LRRK2 are the most common inherited defect relating to development of Parkinson's disease (Bardien *et al.*, 2011; Ross *et al.*, 2011; Wu *et al.*, 2012). Because LRRK2 is an autosomal dominant gene correlating with late onset of the disorder, a mutation in a single allele would be sufficient to stimulate signaling mechanisms responsible for generating Mn toxicity even in the absence of overt signs of Parkinson's disease.

LRRK2 is a relatively complex gene which encodes a large multidomain protein that includes a Rho/Ras-like GTPase (termed Roc or Ras) and a protein kinase domain. Because of this complexity, the mechanism by which mutations in LRRK2 elicits Parkinsonism is likely to be multifaceted as it has been reported to influence mitochondrial function (Saha *et al.*, 2009), protein homeostasis (Tong *et al.*, 2010) as well as a number of signaling pathways within neurons (Habig *et al.*, 2008). Mutations in LRRK2 can also disrupt dopamine (DA) function and lead to a decrease in the DA transporter, DAT, (Adams *et al.*, 2005; Nandhagopal *et al.*, 2008) as well as impair DA-stimulated neurotransmission (Tong *et al.*, 2009). This latter observation is particularly relevant to Mn toxicity as Mn also perturbs dopaminergic function (Peneder *et al.*, 2011; Sriram *et al.*, 2010) and inhibits amphetamine-induced DA release (Guilarte *et al.*, 2008). Thus, LRRK2 displays a number of critical functions many of which can affect or be affected by Mn. Recent studies have shown the presence of multiple genetic mutations in different regions of LRRK2 including those related to decrease kinase activity as being linked to the susceptibility to develop both familial and sporadic Parkinson's disease (Bardien *et al.*, 2011; Ross *et al.*, 2011; Wu *et al.*, 2012). Thus, in this paper we examine the effect that down regulation of LRRK2 has on Mn and DA induced toxicity in HEK cells transfected with the DAT.

2. Materials and methods

2.1 Materials

Human embryonic kidney (HEK 293) cells were purchased from American Type Culture Collection (A.T.C.C.) and HEK-YFP-DAT cells were a gift from Dr. Habibeh Khoshbouei. University of Florida. Dulbecco's Modified Eagle Medium (DMEM), Opti-MEM I reduced serum media, penicillin/ streptomycin, puromycin and fetal bovine serum were purchased from Invitrogen (Gibco BRL, Grand Island, NY). LRRK2 shRNA was obtained from Open Biosystem (Huntsville, AL) and Qiagen Plasmid Maxi Kit from Qiagen (Valencia, CA). Lonza Nucleofector and the Nucleofector Kit were acquired from Lonza (Basel, Switzerland) and AllPrep[®] DNA/RNA/Protein Mini Kit from Qiagen (Valencia, CA). Primary p38 MAP kinase, phospho-p38 MAP kinase, SAP/JNK, phospho-SAP/JNK (Thr183/ Tyr185), and β -actin antibodies for Western blots were obtained from Abcam (Cambridge, MA). Secondary anti-rabbit and anti-mouse antibodies were purchased from Cell Signaling (Danvers, MA) and Western lightning plus substrate from Perkin Elmer (Waltham, MA). CellROX Deep Red Reagent for ROS detection was obtained from Invitrogen Life Technologies (Grand Island, NY) and LRRK2 and GAPDH qPCR forward and reverse primers from Integrated DNA Technologies (IDT).

2.2 Cell culture conditions

HEK-293 and HEK-YFP DAT were maintained in DMEM containing 10% fetal bovine serum and penicillin (50 U/ml) and streptomycin (50 μ g/ml). Stably transfected constructs for both cell lines were maintained in DMEM containing 10% fetal bovine serum and penicillin (50 U/ml), streptomycin (50 μ g/ml) and puromycin (80 μ g/ml). Cells were grown at 37°C in humidified atmosphere containing 5% CO₂ and passaged every 2–3 days at approximately 70% confluency. The concentrations of Mn used in these experiments are similar to that reported previously in the literature (Marreilha dos Santos *et al.*, 2008; Roth *et al.*, 2000; Sidoryk-Wegrzynowicz *et al.*, 2010; Walowitz and Roth, 1999).

2.3 Preparation of shRNA transfectants

The shRNA lentiviral vector used for LRRK2 down-regulation was purchased from Open Biosystems (Thermo Scientific) as Human GIPZ lentiviral shRNAmir individual clone, clone ID V3LHS_644165. The shRNA mature sense and anti-sense sequence for the NS and LRRK2 is presented in Table 1. The shRNA lentivirus was inserted into a pGIPZ vector, which contains a CMV promoter and a TurboGFP marker that provides a marker for shRNA expression in cell culture. The lentiviral shRNA contains bacterial resistance to ampicillin and zeocin as well as a puromycin mammalian selection marker (PuroR) which was used to select for cells expressing the shRNA transcript. Approximately 5 μ g of DNA plasmid containing either the NS or shRNA LRRK2 plasmid was used for transfection into control and DAT containing HEK cells using the Nucleofector kit and the appropriate methods described by Lonza (4D-Nucleofector System).

After the HEK cells were transfected, cell selection was maintained using 80 μ g puromycin and the GFP positive cells isolated using a flow cytometer. Individual colonies were isolated using Scienceware Cloning Discs and the isolated colonies were expanded and maintained in growth media consisting of DMEM, 10% FBS, 1% penicillin/streptomycin, and 80 μ g/ml puromycin. The individual colonies were quantitated for the presence of LRRK2 by RT-qPCR.

2.4 Quantitative PCR

To quantitate the levels of LRRK2 mRNA, approximately 4×10^6 of the transfected cells containing either the NS or shRNA LRRK2 construct was used. Total RNA concentration

isolated was determined using a Nanodrop 2000c spectrophotometer. A mix consisting of 1 µg RNA, LRRK2 Forward Primer, LRRK2 Reverse Primer, Reverse Transcriptase, and RNase/DNase-free water was prepared using Bio-Rad iScript cDNA Synthesis Kit and placed in an Eppendorf Thermal Cycler for cDNA synthesis. (Sequence for LRRK2 forward primer: 5'-GGGAGCAGATGC CAATCAAGCAAA -3', sequence for LRRK2 reverse primer: 5'-AGCTGATGATCTGGCTGTCACCTT-3'. Sequence for GAPDH forward primer: 5'-GGCAGTTGACCAACACAATGCAGA -3'. Sequence for GAPDH reverse primer: 5'-ACCAAATCCGTTGACTCCGAC CTT-3'.) After the cDNA synthesis was complete, the samples were removed from the thermal cycler and stored overnight at 4°C. The cDNA samples were returned to room temperature and added to Bio-Rad iQ™ SYBR® Green Supermix. The samples subsequently inserted into the Bio-Rad iCycler iQ Real Time PCR machine and LRRK2 gene expression was obtained and compared between cells possessing either the non-silencing plasmid to that containing the shRNA construct.

2.5 Cell viability assays

MTT stock was prepared in deionized water at a concentration of 10 mg/ml. Approximately 1×10^5 cells were seeded and grown in six well plates containing DMEM media for 24 hrs. at which time MnCl₂ and/or DA were added to the appropriate wells and incubated for varying periods of time. To assess cell viability, media was removed and the cells washed with 500 µl and 1 ml of OptiMem media after which two ml of OptiMem and 200 µl of MTT dye were added and incubated for an additional 2–4 hours. Following the incubation, the media was removed and 500 µl of isopropanol was added to each well. Aliquots of 150 µl from each well were transferred to a 96-well plate and the absorbance was assessed using a BioTek plate reader/spectrophotometer at 560 nm using Gen5 Microplate Reader Software. The percentage of viable cells was calculated by dividing the untreated samples by the treated samples.

2.6 Reactive oxygen species detection

Approximately 2.5×10^4 cells in each well of a 12-well plate were incubated for 24 hrs. prior to addition of MnCl₂ or DA. After three hrs., CellROX Deep Red Reagent dye was added to each well and cultures were incubated for additional one hr. Following the incubation, fluorescent intensity was analyzed in a fluorometer at 640 nm excitation and 665 nm emission. The percentage increase in ROS activity was determined by dividing the treated samples by the controls which were not treatment with MnCl₂ or DA.

2.7 Western blots

Approximately 4×10^6 cells were grown in 100 mm cell culture plates for 24 hrs. prior to the addition of MnCl₂. Cells were grown for an additional 18 hours at which time the cells were washed with PBS and 150 µl of cell lysis buffer containing protease/phosphatase inhibitor cocktail was added to each plate. Cells were collected and the suspended mixture was transferred to a pre-chilled microcentrifuge tubes which were incubated on ice for one hr. followed by centrifugation at $10,000 \times g$ for 10 min. at 4°C. The supernatant solution was removed and assayed for protein determined by the BCA protein assay. An aliquot of the cytosolic fraction containing 50 µg of protein from each sample was subjected to electrophoresis on a 12% (w/v) polyacrylamide gel and the separated bands were subsequently transferred onto nitrocellulose membranes. The immuno-blotting was performed on SNAP-ID (Millipore) using rabbit primary p38 MAP kinase, phospho-p38 MAP kinase, SAP/JNK, phospho-SAP/JNK (Thr183/ Tyr185) and mouse primary β-actin antibodies. Anti-rabbit IgG conjugate to horseradish peroxidase was used as the secondary antibody and anti-mouse β-actin was used as a loading control. Blots were analyzed by measuring band intensity using Image J software. Overall changes in active phosphorylated

kinase expression and the effect of Mn treatment were determined by comparing band densities in untreated NS and shRNA transfected cultures corrected for β -actin staining.

2.8 Statistical analysis

Data presented are expressed as mean $\pm$ standard error of three or more independent experiments. Where appropriate statistical analysis was based either on two factor ANOVA with replicates or students t-test. The results were considered statistically significant with $p < 0.05$.

3.0 Results

3.1 LRRK2 down-regulation

Application of selective down-regulated of LRRK2 in both control HEK and HEK-YFP-DAT cells allows the examination of the influence of this Parkinson-related gene on Mn toxicity in the presence and absence of DA. To obtain cells in which LRRK2 expression is decreased, transfection with several shRNA constructs were attempted. As indicated by the data presented in Fig. 1, nucleofection with shRNA construct 165 to LRRK2 resulted in successful knockdowns of the kinase in both cell lines based on RT-qPCR analysis. A 75% knock-down was achieved in the DAT cells using this construct (termed DAT 165 cells), and a 71% knock-down in the HEK293 cells using the same construct (termed HEK 165 cells) compared to the NS transfected cells, respectively.

3.2 Mn toxicity

Initial studies were conducted to determine the effect of Mn treatment on cell viability using control NS transfected cells compared to cells containing down-regulated LRRK2 expression measured 24 hrs. after exposure. Results of these experiments with the NS and 165 HEK transfected cell lines shown in Fig. 2A reveal a concentration dependent decrease in cell viability upon treatment with Mn in both transfected cell lines. The percent toxicity obtained is similar to that reported previously for Mn in a number of cell lines (Hirata *et al.*, 2001; Roth *et al.*, 2012a; Stredrick *et al.*, 2004). The data also reveal that the HEK 165 cells displayed a significantly greater reduction in Mn-induced cell viability compared to the control HEK NS cells ($p < 0.0001$) thus, demonstrating that LRRK2 down-regulation potentiates Mn toxicity. The same Mn-treatment was also performed using both DAT NS and DAT 165 cell and results illustrated in Fig. 2B similarly reveal a significant difference in cell viability between DAT NS and DAT 165 cells ($p < 0.0001$). These data clearly demonstrate that down-regulation of LRRK2 results in an increased sensitivity to Mn treatment regardless of the presence of DAT.

The effect time on Mn-induced toxicity was also examined in the DAT containing HEK cells as illustrated by the data presented in Fig. 3. The data reveal there is a significant difference ($p < 0.0001$) in cell viability between DAT NS and DAT 165 cells. At 6 hours, Mn inhibited DAT NS cell viability by approximately 10% and DAT 165 cells by roughly 25%. At 12 hours and 24 hrs., DAT NS cell viability was suppressed by around 35% and 50%, and in DAT 165 cells by almost 55% and 70%, respectively. These data further establish that LRRK2 knockdown DAT 165 cells display a greater sensitivity upon treatment with Mn than the control NS transfected DAT cells.

3.3 DA toxicity

Because the parent HEK cell line lacking DAT is incapable of transporting DA and fail to display toxicity with DA (unpublished observations), the effect of LRRK2 down-regulation on DA toxicity was only examined in the DAT expressing cells. Conditions used in these experiments are similar to that used to assess Mn toxicity described above. Results of these

studies presented in Fig. 4 indicate a concentration dependent decrease in cell viability with DA in both the DAT NS and DAT 165 cells after 24 hrs. of treatment with 50 μ M and 100 μ M DA. As with Mn, these data also reveal there was a significant difference ($p < 0.0001$) in cell viability between DAT NS and DAT 165 cells. At 50 μ M DA, DAT NS cells showed approximately 20% inhibition of cell viability and the DAT 165 cells around 35% inhibition. At 100 μ M DA, the values were around 45% and 75%, respectively. These findings clearly indicate that the LRRK2 down-regulated cells are more sensitive to the DA treatment than the control DAT NS cells.

3.4 Co-incubation of Mn and DA

Based on the observation described above that both Mn and DA are toxic to the transfected DAT expressing cells, it was of interest to examine the combined effect on Mn and DA on cell viability. For these experiments both DAT NS and DAT 165 cells were treated for 24 hours with 50 μ M Mn only, 50 μ M DA only, and 50 μ M Mn plus 50 μ M DA added together. Similar to that reported above, there was a significant increase in cell toxicity comparing DAT 165 cells and DAT NS cells at both 50 μ M Mn ($p < 0.0001$) and at 50 μ M DA ($p < 0.0001$) (see Fig. 5). A similar trend was also observed for the combined treatment regimen ($p < 0.0005$). The results further reveal that toxicity appeared to be partially additive when the two agents were combined. At 50 μ M, Mn inhibited DAT NS cells viability by approximately 17% and DAT 165 cells by 39%. At 50 μ M DA only, DAT NS cells displayed 16% inhibition and DAT 165 cells 36% inhibition whereas the combined treatment resulted in 57% inhibition with the DAT NS cells and approximately 80% inhibition with the DAT 165 cells.

3.5 Reactive oxygen species formation

Prior studies have shown there is an increase in formation of reactive oxygen species (ROS) upon treatment with either Mn or DA (Aboud *et al.*, 2012; Roth *et al.*, 2012b; Stredrick *et al.*, 2004). Based on the fact that the previous experiments described above suggest DAT 165 cells are more sensitive to both Mn and DA, experiments were performed to determine whether increases in ROS may have contributed to enhanced toxicity observed in the LRRK2 knockdown cells. For these experiments, DAT NS and DAT 165 cells were treated for 3 hrs. with varying concentrations of Mn and DA. Results of these experiments, as illustrated by the data presented in Fig. 6, reveal an increase in ROS generation in both cell lines though the increase was greatest at the lower concentrations of both Mn and DA possibly suggesting that the sensitivity of the assay or the amount of ROS produced was at its maximum level at the higher concentrations used. ROS activity was not detected in non-treated cells or at lower concentrations of either Mn or DA. As anticipated, there was a significantly greater increase in ROS formation upon Mn treatment in the DAT 165 cells when compared to DAT NS cells ($p < 0.02$). There was also a significant increase in ROS production upon DA treatment in DAT 165 cells compared to DAT NS cells ($p < 0.04$). These results demonstrate that oxidative stress produced by both DA and Mn treatments likely contribute to cell toxicity and that the DAT 165 cells possessing decreased expression of LRRK2 generate greater increases in ROS when compared to that in control DAT NS cells.

3.6 Phosphorylation of p38

Numerous studies have suggested that Mn toxicity occurs via an apoptotic mechanism (Gavin *et al.*, 1999; Hirata *et al.*, 2004; Roth and Garrick, 2003). Thus, it was of interest to examine the effect of LRRK2 down-regulation on several apoptotic markers associated with Mn toxicity. Initial studies were performed to examine the effect of Mn on p38 activation in both the NS and LRRK2 down-regulated DAT 165 cells. For these experiments, DAT NS and DAT 165 cells were treated with 300 μ M Mn for 18 hours, and the active

phosphorylated form of p38 was detected using Western blot analysis. As indicated by the data presented in Fig. 7A and B, Mn treatment surprisingly resulted in a decrease in phosphorylated p38 ($p < 0.002$) in the LRRK2 down-regulated cells when compared to the NS transfected cells. There was almost a five-fold increase in p38 phosphorylation in the NS transfected cells whereas only a 2.4 fold increase was observed in the DAT 165 cells. The unexpected finding of decreased p38 phosphorylation in the LRRK2 knock-down cells is consistent with prior studies suggesting that LRRK2 may be involved in phosphorylation of p38 (Kim *et al.*, 2012).

3.7 Phosphorylation of JNK

In addition to phosphorylation of p38, studies were also performed to assess the effect of Mn on activation of JNK in the two transfected HEK cell lines. For these experiments, cells were treated with 300 μ M Mn for 18 hours and phosphorylated and non-phosphorylated forms of JNK were assessed by Western blot. As revealed by the data in Fig 8 and Table 2, Mn treatment produced a significant increase in p-JNK expression in the LRRK2 knockdown cells when compared to the DAT 165 NS cells ($p < 0.01$). The increase in JNK phosphorylation in the NS transfected cells was approximately 3-fold whereas that for the DAT 165 cells was greater than 10-fold consistent with greater oxidative stress in the LRRK2 down-regulated cells. Despite this difference, the final levels of phosphorylated JNK were similar in the two cells lines implying that much of this apparent increase may have been caused by the fact that endogenous levels of phosphorylated JNK in the DAT 165 was 70% lower than in the control NS cells.

4.0 Discussion

The overlap in symptoms between Parkinson's disease and manganism suggests they may share similar biochemical deficits which are responsible, at least in part, for both disorders. This is not totally unexpected as the origin of both disorders lies within the basal ganglia and are therefore, not mutually exclusive. In this regard, there is increasing evidence that susceptibility to develop Mn toxicity may be associated with genes that induce early and late-onset of Parkinson's disease. Since autosomal dominant missense mutations in LRRK2 are the most common recognized cause for the development of late onset of Parkinson's disease, its role in the development manganism has to be considered as a plausible biochemical defect linking the two disorders.

As noted in the introduction, LRRK2 is a multidimensional gene encoding a relatively large protein that includes a Rho/Ras-like GTPase moiety (termed Roc, for Ras) and a serine/threonine kinase domain with sequence homology to MAPKKK (Gloeckner *et al.*, 2009; Greggio *et al.*, 2009; Zimprich *et al.*, 2004). Because of this complexity, the mechanism by which mutations in LRRK2 elicits Parkinsonism is likely to be multifaceted and is anticipated to influence a number of signaling pathways. For example, microarray analysis has indicated that down-regulation of LRRK2 can lead to changes in genes involved in actin cytoskeleton-related processes which are known to influence neurite outgrowth, synaptic plasticity, and axonal/dendritic transport (Habig *et al.*, 2008). Other studies have reported that LRRK2 deficiency may act via its inhibition of mitochondrial function (Saha *et al.*, 2009) or even possibly play an essential role in regulating protein homeostasis during aging leading to impairment of protein degradative pathways (Tong *et al.*, 2010). In regard to DA function, LRRK2 can also cause a decrease in the DA transporter, DAT, (Adams *et al.*, 2005; Nandhagopal *et al.*, 2008) as well as impair DA-stimulated neurotransmission (Tong *et al.*, 2009). These observations are particularly relevant to Mn toxicity as Mn can also perturb dopaminergic function (Peneder *et al.*, 2011; Sriram *et al.*, 2010) and inhibit amphetamine-induced DA release (Guilarte *et al.*, 2008) as well as promote mitochondria degeneration. Interestingly, Mn can increase the activity of the most prevalent mutant form

of LRRK2, G2019S, but inhibit wild-type kinase activity which is normally stimulated by Mg (Covy and Giasson, 2010; Lovitt *et al.*, 2010). Thus, LRRK2 displays a number of critical functions many of which can affect or be affected by Mn.

Several different mutations in LRRK2 have been identified and, although some mutations promote increased activity, there are others that, in contrast, impair activity. For example, LRRK2 mutations, R1441C and R1441G, have been shown to affect the GTPase domain and decrease GTPase activity (Santpere and Ferrer, 2009), whereas, the R1941H and G2385R mutations suppress auto-phosphorylation as well as phosphorylation of myelin basic protein and moesin (Jaleel *et al.*, 2007). Similarly, mutation G2385R inhibits kinase activity (Cookson, 2012) whereas mutations in the LRR (leucine-rich repeat) domain can interfere with LRRK2-protein interactions and inhibit activity (Vancraenenbroeck *et al.*, 2012). Because many of the mutations attenuate activity, the studies presented in this paper focus on the effect of down-regulation of LRRK2 on Mn and DA toxicity. To accomplish this we utilized HEK293 cells and HEK293 cells transfected with the DAT which allowed us to regulate and compare the effects of internal and external DA on Mn toxicity in these cell. Since HEK293 cells express the neurofilament subunits, NF-L, NF-M, NF-H, and α -internexin as well as many other proteins which are typically found in neurons, they represent a reasonable model to examine the effect of LRRK2 down-regulation on both DA and Mn toxicity (Shaw *et al.*, 2002). Application of the HEK293 cells have been extensively employed to assess the mechanism by which mutations in LRRK2 affect cell viability and related biochemical processes provoking cell degeneration (Carballo-Carbajal *et al.*, 2010; Taymans *et al.*, 2011). In fact, the parent HEK cells have previously been used to demonstrate that the Parkinson disease related gene, ATP13A2, is capable of protecting against Mn toxicity (Tan *et al.*, 1991).

Initial studies presented in this paper focused on examining the effects of Mn and DA on NS and LRRK2 shRNA transfection in both HEK and DAT HEK cells. As indicated by the results, down-regulation of LRRK2 potentiates cell toxicity upon exposure to Mn in both cell lines when compared to control cells. Since prior studies have demonstrated that the parent non-transfected HEK293 cell line is incapable of transporting DA, further toxicity studies using DA were performed only with the DAT expressing cells. As with Mn, results of these studies demonstrate increased sensitivity towards DA in cells that expressed lower levels of LRRK2 (DAT 165 cells) when compared to that with NS transfected cells.

Although dopaminergic cells within the substantia nigra pars compacta appear to survive the initial insult produced upon protracted exposure to Mn (Stanwood *et al.*, 2009; Sziraki *et al.*, 1998), there are indications that the basic functioning of these neurons is, in fact, disrupted as seen by the observation that release of DA in the striatum is reduced (Guilarte, 2011; Guilarte *et al.*, 2008; Peneder *et al.*, 2011; Sriram *et al.*, 2010). Since Mn exposure can lead to dopaminergic dysfunction, we also investigated the combined effect Mn and DA treatments on cell survival in the LRRK2 down-regulated cells compared to the NS transfected DAT HEK cells. Results of these experiments suggest an additive toxic response in both cell lines. Moreover, the inhibition response when Mn and DA were combined was also statistically greater in the cells expressing decreased levels of LRRK2. These data suggest LRRK2 is an essential protein kinase involved in preventing cell death induced by either Mn or DA and thus, for the first time, implicates it in promoting susceptibility to acquire manganism.

The generally accepted mechanism controlling Mn-induced cell death relates to its disruption of mitochondrial function provoking down-stream oxidative stress-related processes which ultimately manifests in apoptotic cell death (Gunter *et al.*, 2009). Excess exposure to Mn can lead to induction of mitochondrial membrane transients resulting in the activation of the permeability transition pore as well as interference of oxidative

phosphorylation (Brouillet *et al.*, 1993; Chen and Liao, 2002; Galvani *et al.*, 1995; Gavin *et al.*, 1999; Latchoumycandane *et al.*, 2005; Roth *et al.*, 2000; Schrantz *et al.*, 1999; Zhang *et al.*, 2005). Mn-induced disruption of mitochondrial activity stimulates formation of ROS which presumably is one of the initial stress inducing events leading to apoptosis. Since DA has also been reported to generate ROS, the results reported in this paper that the combined Mn and DA treatment potentiate ROS production in both control and LRRK2 down-regulated cells is not unexpected. Most importantly, the generation of ROS was significantly increased in the LRRK2 down-regulated cells in comparison to that seen in the NS transfected cells. This is the first demonstration that LRRK2 deficiency can result in Mn-induced potentiation of ROS formation and thus, may partially explain a mechanism for the observed increase in Mn-induced toxicity in these cells.

As noted above, evidence that apoptosis contributes to Mn toxicity is derived from studies demonstrating stimulation of many of the established biochemical markers and signaling pathways related to programmed cell death which include activation of JNK and p38 kinases (Desole *et al.*, 1997; Hirata *et al.*, 1998; Roth *et al.*, 2000; Schrantz *et al.*, 1999). Since activation of p38 is generally considered to be a hallmark feature of apoptosis, studies were conducted to determine if down-regulation of LRRK2 would similar stimulate increase activation of this kinase when cells were treated with Mn. Although Mn was expected to increase p38 phosphorylation, the response observed was, in fact, opposite in that the Mn-treated DAT 165 cells displayed significantly lower phosphorylated p38 expression. These findings are, in fact, consistent with those previously reported (Kim *et al.*, 2012) in that the levels of phosphorylated p38 were attenuated after lipopolysaccharide treatment in LRRK2 down-regulated HEK293 cells when compared to that in parental and control cells. These findings are also consistent with several reports in the literature suggesting that LRRK2 is a necessary kinase that is involved in phosphorylating p38 and thus, likely accounts for the decrease activity observed in the DAT 165 cells deficient in LRRK2 (Gloeckner *et al.*, 2009).

As noted above, JNK is another protein kinase that has previously been reported to be stimulated in response to Mn as well as by a variety of stress inducing agents (Hirata *et al.*, 1998). In contrast to p38, as noted above, JNK phosphorylation was increased in HEK293 cells upon treatment with LPS, however, the extent of this increase was unaffected by expression of LRRK2 (Kim *et al.*, 2012). Our findings also demonstrate an increase phosphorylation of JNK upon treatment with Mn but, in contrast to LPS treatment, the data suggest there is a relative increase in the LRRK2 down-regulated cells in comparison to that in the NS cells. To a large extent, the percent increase observed was mainly caused by the fact that the endogenous levels of phosphorylated JNK were lower in the 165 cells compared to that in the controls thus, causing the relative apparent increase in phosphorylated JNK. The actual final levels of the phosphorylated JNK species assessed was essentially identical between the two cell lines suggesting that the levels of LRRK2 may, in essence, have had little influence on total JNK activation which is, actually, similar to the conclusions previously reported by Kim *et al.* (Kim *et al.*, 2012)

The studies conducted herein reveal that altered LRRK2 activity not only plays an essential role in development of Parkinson's disease but, in addition, may play a pivotal role in the development of manganism. Results confirm that when LRRK2 is silenced with shRNA both Mn and DA can potentiate oxidative stress and provoke cell death compared to the control HEK293 cells. When LRRK2 is down-regulated, activation of both ROS and JNK is increased whereas phosphorylation of p38, a down-stream substrate for LRRK2, is decreased. These data suggest that down-regulation of LRRK2 may play a predominant role in provocation of Mn toxicity in vivo and thus, joins several other Parkinson's disease markers as potential biochemical processes which may regulate susceptibility to develop Mn

toxicity. Unlike the other genes associated with Mn toxicity, LRRK2 is an autosomal dominant gene and therefore, potentially differentiates a population of individuals that upon exposure to excess Mn can develop early symptoms of manganism even in the absence of overt signs of Parkinson's disease.

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Abbreviations used

LRRK2	Leucine-rich repeat kinase 2
DAT	dopamine transporter
DA	dopamine
Mn	manganese
HEK	human embryonic kidney
ROS	reactive oxygen species
JNK	c-Jun N-terminal kinases
p38	38 mitogen-activated protein kinase
AP13A2	probable cation-transporting ATPase 13A2
pink1	PTEN-induced putative kinase 1
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
DMEM	Dulbecco's Modified Eagle Medium

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Highlights

Transfection of an shRNA to LRRK2 in HEK cell results in decreased expression of the kinase.

LRRK2 down-regulation potentiates both Mn- and dopamine-induced toxicity in HEK cells previously transfected with the dopamine transporter.

LRRK2 down-regulation stimulates Mn-induced oxidative stress as assessed by increases in ROS production and JNK phosphorylation.

LRRK2 down-regulation results in a decrease in p38 activation consistent with LRRK2 playing a role in phosphorylation of this kinase.

Fig. 1. Quantitative PCR results of LRRK2 knockdown in HEK293 and YFP-DAT cells. LRRK2 mRNA expression in HEK and DAT cells transfected with non-silencing (NS) shRNA and shRNA (#165) to LRRK2. Data presents the mean $\pm$ S.E.M. for three independent experiments.

A

B

Fig. 2.

Concentration dependence for Mn toxicity in HEK NS and HEK 165 cells. (A) HEK NS and HEK 165 cells were treated with Mn for 24 hours. Relative cell viability was measured by the MTT assay and compared to that obtained in vehicle only control samples. Data presents are the mean $\pm$ S.E for five independent experiments. There was a significant difference between Mn-induced HEK NS and HEK 165 cellular toxicity (ANOVA; $p < 0.0001$). (B) Concentration dependence for Mn toxicity in DAT NS and DAT 165 cells. DAT NS and DAT 165 cells were treated with 50–100 μ M Mn for 24 hours. Toxicity was measured by the MTT assay and compared with the control untreated samples. Data presents the mean $\pm$ S.E.

for five independent experiments. There was a significant difference between Mn-induced NS and DAT 165 cellular toxicity (ANOVA; $p < 0.0001$).

Fig. 3.

Time course for Mn toxicity in DAT NS and DAT 165 cells. DAT NS and DAT 165 cells were treated with 100μM Mn for the times indicated. Toxicity was measured by MTT assay and compared to vehicle only control samples. Data presents the mean $\pm$ S.E. for three independent experiments. There was a significant difference in cellular viability with time between DAT NS and DAT 165 cells (ANOVA; $p < 0.0001$).

Fig. 4. Concentration dependence for DA toxicity in DAT NS and DAT 165 cells. DAT NS and DAT 165 cells were treated with 50 and 100μM DA for 24 hours. Toxicity was measured by MTT assay and compared to the vehicle only control samples. Data presents are the mean $\pm$ S.E. for five independent experiments. There was a significant difference in cell viability between DAT NS and DAT 165 cells (ANOVA; $p < 0.0001$).

Fig. 5.

Mn and DA co-incubation concentration dependence with DAT NS and DAT 165 cells. DAT NS and DAT 165 cells were treated with 50μM Mn, 50μM DA, or 50μM Mn + 50μM DA for 24 hours. Toxicity was measured by MTT assay and compared to the vehicle only control samples. Data represent the mean $\pm$ S.E. for five independent experiments. There was a significant difference in cellular viability between the DAT NS cells for the combined treatment compared to either the Mn or DA treatment alone (T-Test; both $p < 0.0001$). Similarly, a significant difference in cellular viability was seen between in the DAT 165 cells for the combined treatment vs. either the Mn or DA only treatment (T-Test; both $p < 0.0001$). There was also a statistically significant decrease in cell viability for all three treatments when comparing DAT 165 cells to DAT NS cells (T-Test; 50 μM Mn $p < 0.0001$; 50 μM DA $p < 0.0001$; combined $p < 0.0005$).

Fig. 6. Mn-induced stimulation of reactive oxygen species (ROS) in DAT NS and DAT 165 cells. DAT NS and DAT 165 cells were treated with either 300 and 600 μM Mn or 300 and 600 μM DA for 3 hours. Untreated samples were subtracted from the treated samples for DAT NS and DAT 165 cells. ROS formation was detected using CellROX Deep Red Reagent assay and relative values were determined with a fluorometer. Data presents the mean $\pm$ S.E. for three independent experiments. There was a significant difference in ROS formation between DAT NS and DAT 165 cells with Mn treatment (ANOVA; $p < 0.02$). There was also a significant difference between DAT NS and DAT 165 cells with DA treatment (ANOVA; $p < 0.04$). DAT 165 cells showed a significant increase in ROS formation compared to DAT NS cells with Mn and DA treatments.

A **B** **Fig. 7.**

Western blot detection of p38 MAPK phosphorylation in Mn treated DAT NS and DAT 165 cells. DAT NS and DAT 165 cells were treated with 300 μ M Mn for 18 hours. Intensity of the individual bands was determined by Image J. Graph shows percent change in band density for DAT NS and DAT 165 cells relative to untreated conditions. Data presents the mean $\pm$ S.E. for three independent experiments. DAT 165 treated samples showed a significant decrease ($p < 0.002$) in expression of phosphorylated p38 (P-p38) compared to the DAT NS treated samples.

Fig. 8. Western Blot detection of JNK phosphorylation in Mn treated DAT NS and DAT 165 cells. DAT NS and DAT 165 cells were treated with 300 μ M Mn for 18 hours. Average band densities from 3 independent experiments are shown in Table 2.

Table 1

Hairpin sequence for NS shRNA control	
Mature Sense for NS	ATCTCGCTTGGGCGAGAGTAAG
Mature Antisense for NS	CTTACTCTCGCCCAAGCGAGAG
Hairpin sequence for LRRK2 shRNA #165	
Mature Sense for V3LHS_644165	ACGAAAGCGTTGACGATA
Mature Antisense for V3LHS_644165	TATCGTCAACGCTTTTCGT

Table 2

JNK phosphorylation

Cell Type	Mn (μ M)	Mean $\pm$ S.E.	Fold Increase
NS-UT	0	1	
NS-T	300	29.4 $\pm$ 7.4	29.4 $\pm$ 7.4
165-UT	0	0.29 $\pm$.08	
165-T	300	31.6 $\pm$ 6.7	108.9 $\pm$ 12 ^a

Analysis of Western blots showing JNK phosphorylation in DAT-NS and DAT-165 cells treated with 300 μ M Mn for 18 hours. Data presents the mean $\pm$ S.E for three independent experiments and represents percent change in band density determined by Image J for DA- NS and DAT-165 cells relative to untreated conditions with NS cells.

^aThere was a significant difference in the increase of P-JNK expression between DAT-NS and DAT-165 cells (T-test; $p < 0.005$).