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Characterization of p73 and STAT5b genes that are susceptible to manganese exposure in dopaminergic neurons

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Characterization of p73 and STAT5B genes susceptible to manganese exposure in dopaminergic neurons

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To my family

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ABSTRACT

Manganese (Mn) is an essential trace element found in most living organisms. Chronic exposure to Mn has been linked to the pathogenesis of manganism, which displays neurological abnormalities somewhat similar to those associated with Parkinson's disease resulting from dysfunction of the extrapyramidal motor system within the basal ganglia. However, the exact cellular and molecular mechanisms underlying Mn induced neurotoxicity have not been defined. Oxidative stress mediated dopaminergic neuronal apoptosis is considered to be the prime mechanisms of Mn neurotoxicity. Thus, we sought to identify the genes that are altered during Mn exposure and that lead us to elucidate the mechanisms underlying Mn induced neurotoxicity. First, we used the Qiagen mouse apoptosis RT^2 Profiler™ quantitative PCR array system to identify the genes susceptible to Mn exposure. We treated C57 black mice with 10 mg/kg Mn via oral gavage for 30 days. Afterwards, PCR apoptosis array was performed on substantia nigral tissues for 84 genes associated with apoptotic signaling. Interestingly, we found a significant downregulation of the tumor repressor gene *p73* in Mn-treated substantia nigral tissues. Western blot analyses revealed that the p73 isoform protein lacking transactivation domain at N-terminus (ΔNp73) was downregulated from substantia nigral tissues of C57 black mice exposed to 30 mg/kg Mn for 30 days via gavage. To further characterize the functional role of Mn-induced p73 downregulation in Mn neurotoxicity, we examined the interrelationships between the effects of Mn on *p73* gene expression and apoptotic cell death in an N27 dopaminergic neuronal model. Mn exposure to 300 μM downregulated ΔNp73 proteins in N27 dopaminergic neurons in a time-dependent manner, which consistently supports our animal study. We

further determined that protein level of the ΔNp73 was also reduced in primary striatal cultures in a dose-dependent manner. Furthermore, overexpression of ΔNp73 conferred modest cellular protection against Mn-induced neurotoxicity. Secondly, we identified signal transducer and activator of transcription 5b (STAT5B) gene which was downregulated both in a time-dependent and dose-dependent manner during Mn exposure in N27 dopaminergic neuronal cells over 12 h span. However, STAT1 was relatively unaffected during Mn treatment, indicating isoform-specific effect of Mn on STAT5B. Consistent to N27 dopaminergic neuronal cell model, Mn exposure downregulated STAT5B expression in primary mouse striatal culture. Quantitative RT-PCR analyses showed Mn exposure induces downregulation of STAT5B expression at the transcriptional level as well. Moreover, Bcl-2, a well-known downstream target of STAT5B pathway, was also downregulated concomitantly during Mn exposure. Pretreatment of 20 μM Lactacystin failed to protect downregulation of STAT5B indicating STAT5B downregulation was independent of proteasomal degradation pathway. Pre-treatment of N-Acetyl Cystine (NAC) was shown to protect downregulation of STAT5B. In addition, treatment of MPP^+ in N27 cells showed downregulation of STAT5B. These results support the hypothesis that Mn exposure mediates oxidative stress that induces downregulation of STAT5B. Overexpression of STAT5B cells protected N27 cells against Mn-induced neurotoxicity. Furthermore, overexpression of STAT5B protected mitochondria in N27 cells. Downregulation of STAT5B was recapitulated in substnatia nigra of C57 black mice model treated with Mn and MitoPark Parkinson's disesse model. We also present that human lymphocytes show downregulation of STAT5B during Mn exposure, proposing a potential drug candidate for Mn-induced

neurotoxicity and Parkinson's disease patients. Furthermore, we show that Mn exposure suppresses promoter activity of STAT5B in MN9D dopaminergic cells. To characterize the molecular mechanisms underlying STAT5B downregulation during Mn neurotoxicity, we examined the effects of 300 μM Mn exposure for the promoter analysis of STAT5B expression. We subcloned the STAT5B promoter 1 from mouse brain. Analysis of mouse STAT5B promoter from 2,000 nt upstream to 5,00 nt downstream region indicated that a proximal region near exon 1 contains the regulatory element in response to Mn exposure. Detailed mutational analyses of the putative transcription factor binding site revealed that a Sp1 like transcription factor binding sites near exon 1 may be required for the suppression of STAT5B in Mn-induced neurotoxicity. Two KLF binding sites exhibited to be transcription repressor that can respond to Mn exposure, whereas one Sp1 binding sites exhibited transcription activator which senses Mn exposure and reduces its activity. These data suggest Mn exposure alters the profiles of transcription factors to downregulate anti-apoptotic STAT5B signaling via an Sp1-like transcription factor-dependent mechanism in dopaminergic neurons, which may significantly contribute to Mn neurotoxicity. Taken together, our results suggest that Mn exposure compromises the expression of neuroprotective ΔNp73 and STAT5B in dopaminergic neurons for Mn-induced neurotoxicity, thereby exacerbating neuronal cell death (NIH grants ES10586, ES19267, NS74443).

CHAPTER I: GENERAL INTRODUCTION

Dissertation Organization

This dissertation is written in an alternative thesis format and consists of manuscripts that are being prepared for submission to scientific research journals. The dissertation contains a general introduction, three research papers, a general conclusion that discusses the overall findings from all the chapters, and an acknowledgment. The references for each individual section are listed at the end of the corresponding chapters, except for the general introduction section whose references are listed following the general conclusions. The introduction section under chapter I provides a brief overview of Parkinson's disease, manganese, and the research objective. The background and literature review I provides background information on Parkinson's disease. The background and literature review II provides background information on manganese, protein kinase C delta, p73, and STAT5B with brief overall hypothesis. The manuscript from Chapter 2 was recently submitted to Neurotoxicology and in the process of communication. This chapter identified ΔNp73 was downregulated during Mn exposure and shown to be neuroprotective. Chapter 3 identified signal transducer and activator of transcription (STAT) 5B was downregulated during Mn exposure and characterized its downregulation. Chapter 4 studies the mechanisms of how Mn exposure induces downregulation of STAT5B. Chapter 3 and 4 are in the process of being submitted for publication. This dissertation contains the experimental results obtained by the author during his Ph.D. study under the supervision of his major professor Dr. Anumantha G. Kanthasamy at Iowa State University.

Introduction

Parkinson's disease (PD) is a chronic, progressive, neurodegenerative disorder after Alzheimer's disease in US (companies, 2014). In US, about 1 million people were affected and it is predicted to double the frequency of PD by 2040 (Kowal et al., 2013). PD was first described by James Parkinson in 1817 as the "shaking palsy" as PD patients show movement disorders such as bradykinesia (slowness of movement), resting tremor, rigidity and postural instability. Pathological hallmarks of PD are the loss of dopaminergic neurons in substantia nigra (SN) and Lewy bodies composed of protein aggregates. When PD patients begin to show the symptoms of PD, about 50% of dopaminergic neurons in SN and 80 % of striatal dopamine have been depleted. Average onset of idiopathic PD is 60 years. There are rare occurrences of PD at younger age between 20 to 40 years. Genetic factors play important role in early familial PD. Genes associated with PD include α-synuclein, parkin, PINK-1, LRRK2 and DJ-1 (Kumar et al., 2012, Ben-David and Tu, 2015). However, over 90 % of PD are independent of genetic factors. Environmental factors are believed to play important role in idiopathic PD such as pesticides, industrial byproducts, and metals (Lai et al., 2002). Neurotoxic agent such as MPTP was shown to induce degeneration of dopaminergic neurons (Bove et al., 2005). Paraquat has similar chemical structure to MPTP (Yang and Tiffany-Castiglioni, 2005). Both molecules can easily cross blood-brain barrier and induce dopaminergic neuronal degeneration. Currently, there is no cure to PD. The strategy of PD medicine is to slow down the progress of PD. Levodopa, a precursor of dopamine, is the golden standard medicine in PD (Clarke, 2004).

Manganese (Mn) is a versatile metal used in industry for decades and also an essential transition metal in living organisms. However, overexposure to Mn induces chronic, progressive neurodegenerative disease named as mangansism. The clinical symptoms of manganism are somewhat like that of PD (Farina et al., 2013, Martinez-Finley et al., 2013). Pathological features of Mn-induced neurotoxicity include loss of dopaminergic neurons in midbrain. Major route of Mn intake is through ingestion and under tight regulation of Mn uptake. Inhalation of Mn is another important route for Mn uptake and associated with occupational settings such as welders and miners. Overexposure to Mn induces reactive oxygen species (ROS) and induces oxidative stress (Erikson et al., 2004, Reaney and Smith, 2005, BARBUSIŃSKI, 2009). Oxidative stress is under tight regulation. However, dysregulated oxidative stress activates microglia which in turn induces inflammation to lead neurodegeneration. However, the exact cellular and molecular mechanisms underlying Mn-induced neurotoxicity remain elusive. Hence, identification of novel key molecules in Mn-induced neurotoxicity is essential in understanding Mn-induced neurotoxicity and development of potential drug. The present studies seek to identify novel key molecules and understand the mechanism underling Mn-induced neurotoxicity.

Background and Literature Review I

Parkinson's disease

Parkinson's disease (PD) is chronic progressive neurodegenerative diseases. Parkinson's disease is one of the most common neurodegenerative diseases, second only to Alzheimer's diseases (Nagatsu and Sawada, 2006). In US, about 1 million people were affected and it is predicted to double the frequency of PD by 2040. The prevalence of PD increases to 1% to 2% of population for a person over age of 65 and to 4% to 5% over age of 85, respectively (Kowal et al., 2013). Average onset of PD is 60 years. However, there are rare occurrence of PD at younger individuals between 21 and 40 years. Most PD patients experience 5 to 10 years of severe motor behavioral disorders. Men has higher risk in developing PD than woman (Rubin, 2007). The duration of PD has average of 15 years from diagnosis to death (Lees et al., 2009). In 1817, James Parkinson originally

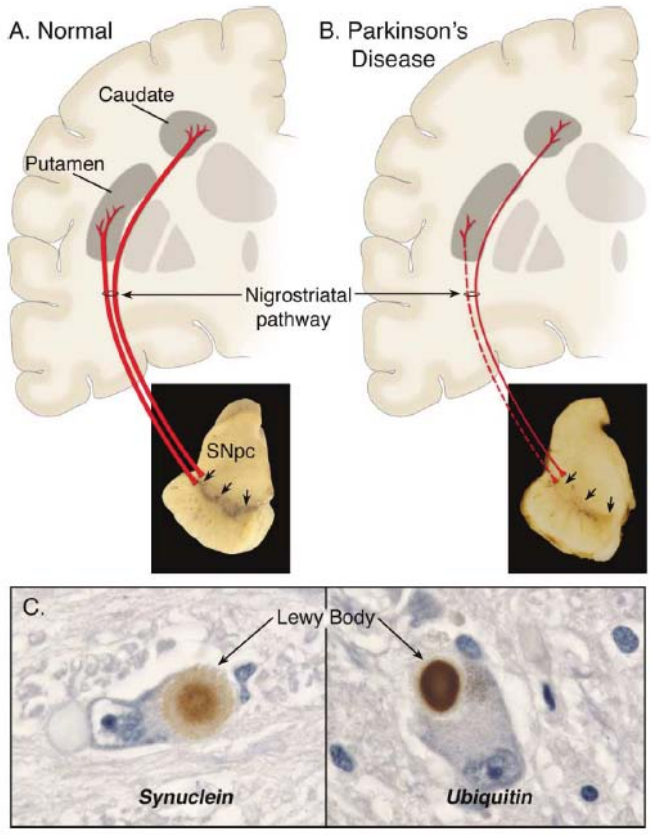

Figure 1. Neuropathology of Parkinson's Disease. This figure is adapted and modified from (Dauer and Przedborski, 2003).

described PD as “shaking palsy” as the main clinical features in 1817. Tremor is one of main clinical features of PD and occurs at rest in the PD patients. When they move voluntarily, tremor decreases. PD patients have rigidity and show resistance to passive movement of limbs. Slow movement was referred as bradykinesia and impairs the quality of life of PD patients as it causes very long time to do in their common lives. Postural or gait instability is another features of PD patients. PD patients also show reduction of amplitude of movement (hypokinesia) and absence of unconscious normal movement (akinesia) (Dauer and Przedborski, 2003). PD patients also have non-motor symptoms which can take place in all stages of PD. Prodomal non-motor symptoms include rapid eye-ball movement sleep behavior disorder, speech abnormalties, constipation, olfactory dysfunction, depressions, gastrointestinal and cognitive deficits. Pathological feature of PD is the loss of dopaminergic neurons in substantia nigra (SN) which results in deficiency of dopamine (DA) in striatal neurons. When PD patients begin to show signs of PD, there are around 80% depletion of striatal dopamine and 50% loss of SN neurons (Cheng et al., 2010). Another hallmark of PD is Lewy bodies composed of aggregated α-synuclein and other degradation products (Dauer and Przedborski, 2003).

Hereditary forms of PD showed that several genes were associated with the pathogenesis of PD (Dauer and Przedborski, 2003). Several hereditary genes found include α-synuclein, LRRK2, Parkin, DJ-1, Uch-L1, and PINK1. However, one genetic studies of PD carried out by Tanner group (Tanner et al., 1999) provided uncertainty of the hereditary genes as the cause of PD. They examined the incidence of PD in monozygotic and dizygotic twins and found indistinguishable concordance rate between monozygotic-dizygotic twins,

suggesting lack of genetic influence and strong support of environmental factors in the etiology of PD. In fact, the vast majority of PD cases over 90% do not have genetic linkage, thus referred as sporadic PD. Several environmental factors were shown to be associated with the etiology of PD such as pesticides, herbicides, gasoline additives, industrial contaminants, metals, and contaminated drugs (Priyadarshi et al., 2000, Ritz and Yu, 2000, Kitazawa et al., 2002, Kanthasamy et al., 2006a, Saminathan et al., 2011, Song et al., 2011, Caudle et al., 2012, van der Mark et al., 2012, Van Maele-Fabry et al., 2012, Ngwa et al., 2014, Charli et al., 2015).

Manganese (Mn) is a transition metal and has been used in industry for decades. Environmental exposure to Mn is known to cause manganism which is a chronic, progressive and neurodegenerative syndrome somewhat like Parkinson's disease. The higher incident of manganism has been observed in among occupational groups including welders, miners, farmers who have been exposed to Mn based fungicides such as Maneb and Mancozeb (Roth, 2009, Racette et al., 2012, Farina et al., 2013, Michalke and Fernsebner, 2014). The usage of drinking waters containing higher level of Mn was reported to have adverse neurological effects (Kondakis et al., 1989). Results from several researches suggested that overexposure to Mn induces cellular changes including depletion of dopamine, increased oxidative stress, and cell death (Kitazawa et al., 2002, Hussain et al., 2006, Fernsebner et al., 2014). However, the exact cellular and molecular mechanisms of how Mn exposure induces dopaminergic neuronal degeneration remain unclear.

Unfortunately, there is no known available cure for PD in spite of advancement of researches on PD in recent years. Many commercial medicines only alleviate the symptoms

of PD. Current golden standard of the PD medicine is levodopa which is a precursor of dopamine and produce dopamine in brain (Clarke, 2004). Treatment of levodopa, however, gives patients some side effects such as such as nausea and vomiting. Long-term motor complications involve abnormal involuntary movements including dyskinesias, dystonia, and motor fluctuations. Moreover, the clinical efficacy of treatment of levodopa often declines as the PD progresses. Several other approaches to provide PD treatment have been made including dopamine agonist, Monoamine oxidasae (MAO) inhibitors, catechol O-methyltrasnferase (COMT) inhibitors, and nerotrophic factors (Factor et al., 2001, Suchowersky et al., 2001, Mandel et al., 2005, Zeng et al., 2006, Schober et al., 2007, Kelley et al., 2012). The economical burden of treating PD patients is huge exceeding $14.4 billion in US, which is $8.1 billion higher than similar population without PD. As the society will have more elderly population in the future, the estimates of medical costs and healthcare bills are expected to increase significantly (Kowal et al., 2013).

In the relatively long history of PD about two centuries, the breakthrough discovery was the finding that dopamine (DA) in the mammalian brain. The loss of neurons in substantia nigra pars compacta (SNpc) leads the deficiency of DA in the striatal neurons which is believed to be the main cause of PD. The finding that the replenishment of DA alleviates many symptoms of PD strongly supports the hypothesis. Regardless of PD patients whether young onset PD with inheritable genes or sporadic forms of PD, loss of DA in striatal neurons and SN leads the PD symptoms. The pathways of dopaminergic neuronal system arise from midbrain including SN, hypothalamus, and ventral tegmentum area (VTA) (Bjorklund and Dunnett, 2007, Van den Heuvel and Pasterkamp, 2008). Nigral neurons

project to the striatum along the nigrostriatal pathway. Briefly, neurons from VTA project to limbic and cortical areas along mesolimbic and mesocortical pathways. Nigrostriatal pathway consists about 70% of the total dopaminergic neurons in the central nervous system (CNS) (Van den Heuvel and Pasterkamp, 2008). Substantia nigra contains very dense population of neurons and contains a darker pigment called neuromelanin. Substantia nigra plays important roles in receiving neuronal inputs and gives efferent signal to striatal neurons. Loss of dopaminergic neurons in nigrostiratal pathway is largely associated with PD because it is associated with dorsal sensorimotor striatum.

The Genetics of PD

Genetic factors can be the causation to PD and has been studied extensively last two decades. This familial PD, however, comprises of only 5 to 10 % of the total PD cases. The genes identified to have causal effects to PD have been assigned to PARK loci. Genes whose mutations have strongly been associated with PD include *SNCA*, LRRK2, Parkin, PINK1 (PARK6), PARK1, PARK3, DJ-1 (PARK7), PARK9, PARK10, PARK11, Tau, and Uchl1-1 (Poulopoulos et al., 2012, Fujioka et al., 2014).

α-synuclein encodes a small pre-synaptic protein (140 amino acid) from the designated gene, *SNCA*. It is one of the isoform of the synuclein family members (Polymeropoulos et al., 1997). α-synuclein has been found to be one of major component in Lewy bodies in PD patient (Spillantini et al., 1997). Mutations of α-synuclein, namely A30P, A53T, and E46K have been associated with German, Greek, and Spanish familial PD, respectively ((Polymeropoulos et al., 1997, Kruger et al., 1998, Zarranz et al., 2004). These

α-synuclein mutant have offered valuable genetic tools to study the genetic model of PD. Recently, four additional mutation in SNCA gene (A18T, A29S, H50Q, and G51D) have been found (Appel-Cresswell et al., 2013, Hoffman-Zacharska et al., 2013, Lesage et al., 2013). Although α-synuclein appears to play very important role in pathogenesis of PD, the incidence of mutation of *SNCA* gene is rare. Additionally, genomic multiplication of the SNCA gene is present in 1-2% of patients with autosomal dominant PD (Ibanez et al., 2009). Triplication of *SNCA* gene produce double amount of α-synuclein, whereas duplication makes 50% more of α-synuclein. Families with duplication of SNCA gene appears to have a more damaged brainstem damage and progress with movement disorders (Fuchs et al., 2007). In line with finding with genomic multiplication of SNCA, overexpression of α-syuclein was

Table 1. Genes and Loci associated with parkinsonism[a]

PARK locus	Gene	Map position	Clinical phenotype	Pathology
PARK1/4	*SNCA*	4q21	Parkinsonism with common dementia	Lewy bodies
PARK2	*parkin*	6q25-q27	Early-onset, slowly progressing parkinsonism	Lewy bodies
PARK3	Unknown	2p13	Late-onset parkinsonism	Lewy bodies
PARK5	*UCHL1*	4p14	Late-onset parkinsonism	Unknown
PARK6	*PINK1*	1p35-p36	Early-onset, slowly progressing parkinsonism	One case exhibiting Lewy bodies
PARK7	*DJ-1*	1p36	Early-onset parkinsonism	Unknown
PARK8	*LRRK2*	12q12	Late-onset parkinsonism	Lewy bodies (usually)
PARK9	*ATP13A2*	1p36	Early-onset parkinsonism with Kufor–Rakeb syndrome	Unknown
PARK10	Unknown	1p32	Unclear	Unknown
PARK11	*GIGYF2*	2q36-q37	Late-onset parkinsonism	Unknown
PARK12	Unknown	Xq	Unclear	Unknown
PARK13	*Omi/HTRA2*	2p13	Unclear	Unknown
PARK14	*PLA2G6*	22q13.1	Parkinsonism with additional features	Lewy bodies
PARK15	*FBX07*	22q12-q13	Early-onset parkinsonism	Unknown
PARK16	Unknown	1q32	Late-onset parkinsonism	Unknown
FTDP-17	*MAPT*	17q21.1	Dementia, sometimes parkinsonism	Neurofibrillary tangles
SCA2	*Ataxin 2*	12q24.1	Usually ataxia, sometimes parkinsonism	Unknown
SCA3	*Ataxin 3*	14q21	Usually ataxia, sometimes parkinsonism	Unknown
Gaucher's locus	*GBA*	1q21	Late-onset parkinsonism	Lewy bodies

[a] Adapted and modified from (Martin et al., 2011).

shown to induce neurotoxicity with a dopaminergic neuronal loss, which can be rescued with overexpression of Rab1 (Cooper et al., 2006). Increased amount of α-synuclein was shown to tend oligomerization (Lee et al., 2002). Dopamierngic neuronal cells with overexpression of α-synuclein was showe to be more vulnerable to the exposure to neurotoxic insult of dieldrin (Kanthasamy et al., 2005). Although the exact function and role of α-synuclein is largely not known, the PD patients with mutations in SNCA genes have clinical and pathological features similar to those observed in sporadic PD. They also responded well with levodopa treatment. Inclusion of α-synuclein has been found not only in PD but also in other neurodegenerative diseases such as Alzeheimer's disease, Lewy body dementia, and multiple system atrophy (Spillantini et al., 1998, Mukaetova-Ladinska et al., 2000, Burn and Jaros, 2001). Many mutations of LRRK2 have been associated with families. LRRK2 mutations show in mid to late onset of PD (Giasson et al., 2006). The pathology from the LRRK2 mutation is very variable giving difficulty in understanding the role of LRRK2 in PD. One missense mutation (G2019S) of LRRK2 was shown to increase the kinase activity of LRRK2 and alters miR activities to regulate gene expression (Greggio et al., 2008, Gehrke et al., 2010). LRRK2 G2019 is relatively common varing between 1% - 40% of PD cases with highest frequency in people of North African descent (Ozelius et al., 2006). The patient with LRRK2 mutations show high incidence to have Lewy body PD.

Parkin was autosomal recessive juvenile PD and causes early onset of PD (Kitada et al., 1998) accounting for 50% of familial cases with age of onset younger than 20 years (Lucking et al., 2000). More than 100 mutations in Parkin have been identified including point mutation, deletion, duplication, and exonic rearrangement. The PD patients with Parkin

mutation respond well to dopaminergic treatment. Parkin is an E3 ubiquitin ligase and plays roles in process of ubiquitination for unnecessary, damage or misfolded proteins (Shimura et al., 2000). Parkin also plays a role in the maintenance of mitochondria (Rothfuss et al., 2009).

DJ-1 is associated with autosomal recessive early onset PD, accounting for 1 – 2 % of early onset PD (Pankratz et al., 2006). DJ-1 gene encodes 189 amino acid protein which functions as a cellular sensor for oxidative stress (Junn et al., 2005). The mutation in the DJ-1 protein causes tendency to misfolded and unstable proteins which gets degraded by proteosome (Anderson and Daggett, 2008). Thus DJ-1 mutation reduces the neuroprotective potentionals (Malgieri and Eliezer, 2008).

The phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1) gene is associated with 1% - 9% of early onset PD (Klein et al., 2005, Kumazawa et al., 2008). Most mutations of PINK1 is missense or nonsense mutation with nearly equal frequencies of all 8 exons (Camargos et al., 2009). PINK1 is a 581 amino acid protein and ubiquitously expressed. N-terminal domain of PINK1 has mitochondrial targeting motif whereas C-terminal domain contains autoregulatory functions. PINK1 and Parkin are found to have functions in a common pathway to maintain healthy mitochondria by sensing and eliminating damaged mitochondria. Stabilized in a lower membrane potential, PINK1 recruits Parkin from cytosol which initiate autophagic clearance of damaged mitochondria (Narendra and Youle, 2011).

Molecular and cellular mechanisms in idiopathic Parkinson's disease

The findings of genetic tools and advanced understanding of the genetic models of PD have expanded extensively and provided deep insight in understanding partial molecular and cellular mechanisms of pathogenesis of PD. However, the exact molecular and cellular

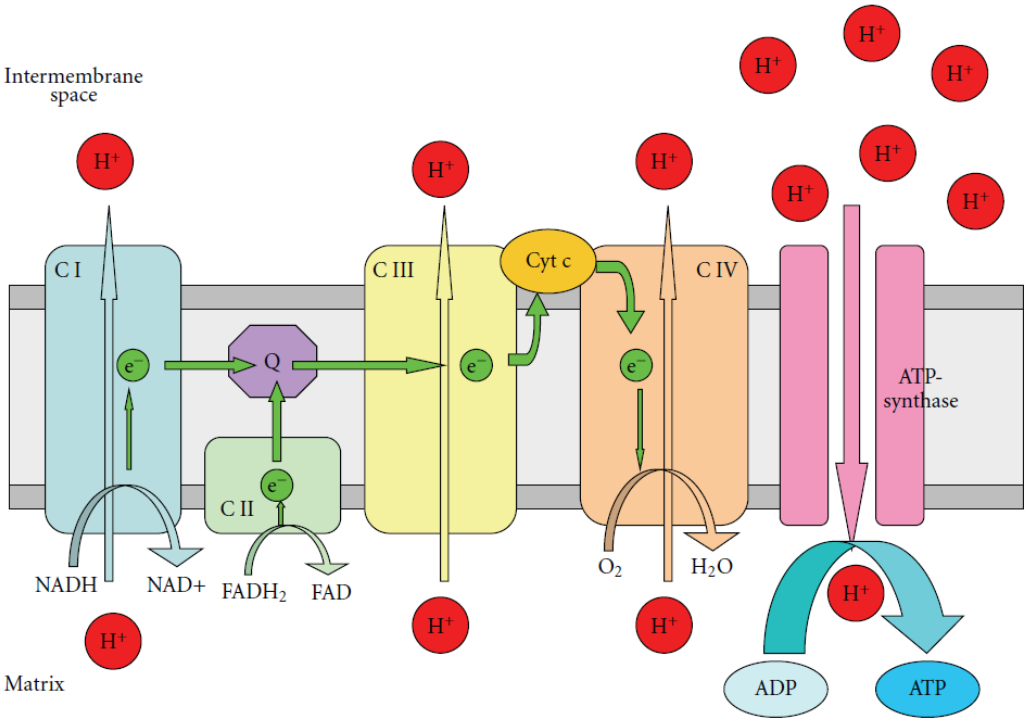

Figure 2. Schematic view of mitochondrial electron transport chain involved in oxidative phosphorylation. Adapted and modied from (Keane et al., 2011).

mechanisms in understanding the underlying mechanisms of idiopathic Parkinsons' disease have been elusive and remain to be studied extensively largely due to the multiple causations to etiopathgenesis of PD. But, recent advancement of studies of PD proposed the oxidative stress mediated apoptosis in affected regions in brain is strongly associated with

etiopathgenesis of PD. Oxidative stress arises from excessive usage of molecular oxygen which is required in many cellular reactions for a catalyst and energy production. These biochemical reactions using oxygen inevitably produce reactive oxygen speces (ROS) such as superoxide anions, hydrogen peroxide, hydroxyl radicals, and peroxy radicals. Besides, reactive nitrogen species (RNS) such as peroxynitrite and nitro-tyrosyl radicals can be produced from the reactions. Although consumption of oxygen and removal of ROS and RNS are tightly regulated, excessive amount of ROS and RNS can be detrimental to cells, even to death (Loh et al., 2006, Miller et al., 2009). To cope with negative impacts of ROS from usage of oxygen, cells utilize antioxidant enzymes such as catalase, superoxide dismutase, and gluthathione peroxidase. Oxidative stress can damage to DNA, protein, and lipids, leading to activation of apoptosis (Miller et al., 2009). Post mortem studies consistently showed increased oxidation of DNA, protein, and lipids in Alzehimer's disease, Parkinson's disease, and other neurodegenerative disease such as stroke, Huntington's disease, and amyotrophic lateral sclerosis (Cardoso et al., 2005, Mariani et al., 2005, Moreira et al., 2005, Miller et al., 2009, Hwang, 2013).

Parkinson's disease patients have multiple sources of oxidative stress from excessive usage of dopamine in substantia nigra, genetics factors to environmental factors. Substantia nigra contains high density of dopaminergic neurons which oxidize dopamine by monoamine superoxide which facilitate the reaction to produce superoxide and hydrogen peroxide (Halliwell, 1992). Although DA is contained in vesicles and regulated tightly, excessive DA gets oxidized spontaneously or via enzymatic reaction to produce DA quinone. DA quinone was shown to modify nucleophiles from nucleic acids and proteins which leads to

dysregulate biochemical reactions and pathways to affect cell fates. In addition, DA quinone can modify α-synuclein monomer to promote the form of cytotoic protofibril form (Conway et al., 2001). DA quinine was shown to inactivate DA transporter and tyrosine hydroxylase, which is rate-limiting key enzyme to produce dopamine (Kuhn et al., 1999). DA quinone can induce dysfunction of mitochondria which leading to generate excessive reactive oxygen species (ROS) (Van Laar et al., 2009). Mitochondria is another big source to generate oxidative stress associated with pathogenesis of PD. Mutations of genes such as parkin, DJ-1, and PINK are associated with familial forms of PD (Muftuoglu et al., 2004, Gandhi et al., 2009, Irrcher et al., 2010). One of the neurotoxicant used to study Parkinson's disease is 1-Methyl-4phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP was the contaminant in the synthetic heroin drug (Schober, 2004, Bove et al., 2005). The drug abusers in 1982 showed neurodegenerative disease that resemble the clinical symptoms of PD such as tremor, rigidity, postural instability, bradykinesia (Schober, 2004). Autopsy results of drug abusers exhibited degeneration of dopaminergic neurons in SN, except Lewy bodies (Bove et al., 2005, Landrigan et al., 2005). MPTP is lipophilic molecule and cross blood-brain barrier. Monoamine oxidase in astrycyte converts MPTP to MPP^+ which is transferred through DA transporter and accumulates in mitochondria (Schober, 2004, Uversky, 2004). High level of MPP^+ in mitochondria inhibits electron transport chain complex I leading to production of ROS (Maguire-Zeiss et al., 2005). Excessive ROS in turn triggered cellular apoptosis through activation of caspase and DNA fragmentation (Kotake and Ohta, 2003). Paraquat (PQ), 1,1'-dimethyl-4,4-bipyridinium, is a widely used herbicide with similar chemical structure to MPP^+ (Chun et al., 2001). PQ can cross blood-brain barrier through neutral amino acid

transporter. PQ is selectively accumulated in dopaminergic neurons in SN through DA transporter, which produce ROS and leads to cell death (Thiruchelvam et al., 2000, Yang and Tiffany-Castiglioni, 2005). These toxicant models have degeneration of mitochondria in common which leads to increase of ROS and cell death.

Microglia are resident immunocompetent cells in brain and one of the four major types of cells in CNS (Mander et al., 2006). The patients of PD have more than six times of microglia compared to control group (McGeer et al., 1988). Microglia responds faster than astrocytes and becomes activated in exposure to damaged or infected cells or immune mediators such as IL-1β or TNF-α (Mander et al., 2006). Exposure to MPTP, rotenone, manganese (Mn) was shown to induce activation of microglia (Liu, 2006, Zhao et al., 2009). Activated microglia can produce superoxide and nitric oxide which contribute to oxidative stress (Qian et al., 2010).

Background and Literature Review II

Manganese

Manganese was first discovered by Sheele in 1774 and given its name by Guyton de Morveau in 1785 to distinguish from magnesium (Inoue and Makita, 1996). Mn is a naturally occurring transition metal element from many types of rocks and soils (Williams et al., 2012b). Mn exists in eleven different oxidation states from minus three to plus seven with most common valences being $^{2+}$, $^{4+}$, and $^{7+}$. The versatile chemical properties allow its use for many industrial uses as well as important biological elements (Michalke and Fernsebner, 2014).

Manganese exists as a component of various minerals in nature. Most Mn ore are consumed in industry to produce ferromanganese which is widely used for the production of various steels such as carbon steel, stainless steel, high-temperature steel. Manganese is also used for the non-metallic purposes. Manganese dioxide is used for production of dry cell batteries, matches, fireworks, and glass-bonding materials. It serves as starting materials for other types of manganese compounds. Manganese chloride is used as catalyst in the chlorination of organic compounds and as essential manganese suppliers in animal diets. Manganese sulfate is used for fertilizers, while potassium permanganate as a disinfectant, anti-algal agent and bleaching. Manganese is used for production of street drugs as well. Manganese has been used as fuel additives such as MMT for decades to replace tetraethyl lead in gasoline. For the medical purpose, mangafodipir trisodium is used for magnetic resonance imaging to improve lesion detection of liver and pancreas (Williams et al., 2012b, Martinez-Finley et al., 2013).

Manganese exposure

Exposure of manganese to human is mainly through inhalation and ingestion (Farina et al., 2013). Plants such as wheat, rice and tea leaves represent a major source of dietary manganese. Drinking water is another major source for manganese intake. Some regions in Bangladesh were reported to have as high as $2.4mg^{-1}$ of manganese in drinking water (Frisbie et al., 2009). Manganese absorbed through gastrointestinal system goes through tight control of homeostasis allowing excretion of excessive manganese (Klaassen, 1974). Inhaled exposure to manganese is frequently related to occupational conditions. Norwegian

manganese alloy producing industry produced as high as 27 mg Mn m^{-3} (Ellingsen et al., 2003). Manganese mining site in Mexico was reported to produce high level of manganese in the air. A range of manganese from 0.04 to 5,86 µg Mn m^{-3} in the air was reported in 2 km distance from the site (Rodriguez-Agudelo et al., 2006). Welding operation often produce

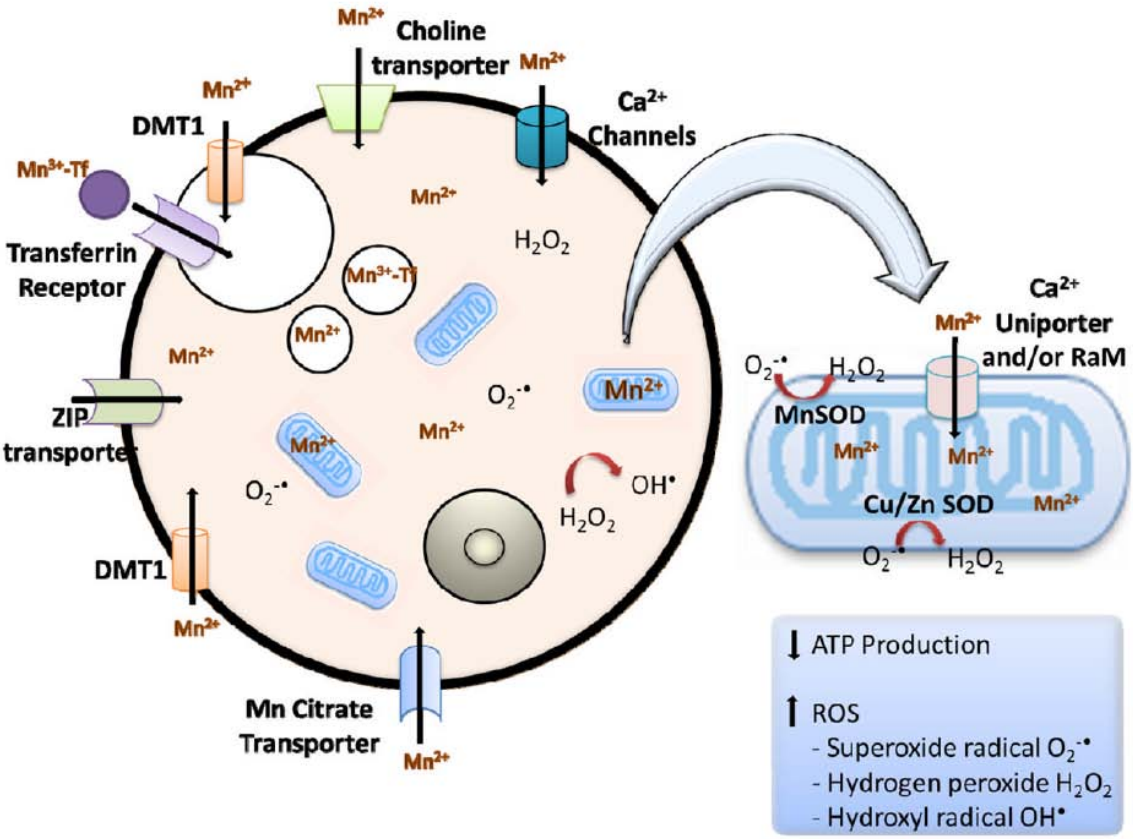

Figure 3. Mn transport. Adapted and modified from (Martinez-Finley et al., 2013).

gaseous particle of manganese. Historically, poorly conditioned workplace without proper ventilation puts higher level of manganese (Antonini et al., 2006, Flynn and Susi, 2009). There are non-occupational sources for manganese exposure to human. Inhalable manganese particles are emitted from manganese containing fireworks and abrasion of steel alloys.

Combustion of fossil fuels emits manganese 5-300mg Mn kg^{-1}. The environmental source for manganese exposure include the gasoline additives, methylcyclopentadienyl manganese tricarbonyl (MMT) was reported to produce manganese from 5 to 20mg kg^{-1} (Pfeifer et al., 2004), and some pesticide such as maneb and mancozeb (Pennington and Wilson, 1990). The use of manganese as medical agents provides another source. Long term parental nutrition was reported to induce Hypermanganesemia (Fell et al., 1996). The majority of manganese intake is taken orally through gastrointestinal systems. A healthy individual absorbs 3 to 5 % of manganese (Davidsson et al., 1989). In bloodstream manganese is bound mainly to transferrin and albumin. Liver is the main organ to eliminate manganese (Madejczyk et al., 2009). As manganese absorbed through gastrointestinal system is under tight homeosystem, adverse effects of ingestion of excess amount of manganese are seldom reported (Portier, 2012). Most inhaled manganese gets deposited in the alveolar or pulmonary region of the respiratory track (Zimmer and Biswas, 2001). Depending on the degree of solubility of manganese aerosol, manganese particulate matter shows different time for its removal (Antonini et al., 2006). Manganese small particles gets also deposited in nasal/head airway region which provides direct route to deliver manganese to brain by bypassing blood-brain barrier (BBB) and homeosystem in liver (Henriksson and Tjalve, 2000). Small size of manganese particle is located in very close proximity to alveolar blood vessel and gain direct access to blood stream. In physiological condition, manganese tends to bind to carrier molecules such as transferin or albumin and get transported to different organs. Molecules associated with Mn transport were depicted in Figure 3. The central nervous system, or CNS, is a particularly important organ that receives an excessive amount of manganese-induced

toxicity. Manganese accumulates in globus pallidus, striatum and the substiantia nigra pas compacta (Inoue et al., 1975). Manganese can travel to brain where influx of molecules is tightly controlled in blood-brain barrier with the help of ligand. Transferin, divalent metal transporter-1 (DMT-1), and citrate were reported to transport manganese (Farina et al., 2013).

Parkinson's disease like manganese-induced neurotoxicity

Chronic exposure of low level of manganese induce to develop neurotoxicity called manganism whose pathological feature is similar to that of parkinson's disease patients (Lucchini et al., 2009, Roth, 2009, Guilarte, 2010, 2011, Caudle et al., 2012). Both patients exhibited movement disorders such as loss of postural stability, gait disturbance and a propensity to fall. Unlike patients of parkinson's disease, manganese exposed patients have no response from L-dopa treatment and resting tremor which is important features of parkinson's disease. Biochemical research revealed that both patients have low level of effects of dopaminergic neurons (Guilarte, 2011). Yet, the pathology of PD is largely attributable to the progressive loss of dopaminergic neurons due to the neurodegneration in SN and STR. The symptoms of manganisms is more related to degeneration of globus pallidus. Manganism patients generally lack Lewy body, a hallmark of PD (Caudle et al., 2012). Yet, exposure to Mn was reported to increase the risk of developing PD (Santamaria et al., 2007). Fifteen welders were reported to have the onset of PD 17 years earlier (Racette et al., 2001). Thus, protection of dopaminergic neurons from neurotoxicity could help both patients.

Cellular and biochemical changes following manganese exposure

Manganese preferentially accumulates in mitochondria when cells were exposed to manganese. Ca^{2+} uniporter appears to transport manganese into mitochondria (Gavin et al., 1999). Mitochondria are the sites where eukaryotic cells generate energy through aerobic

Table 2. Similarities and Diffeences between PD and manganese-induced toxicity[a]

	Parkinson's Disease	Manganese Toxicity
Genetic Determination	Yes, can be autosomal dominant or autosomal recessive. Approximately 10–20% of cases linked to genetic causes	No
Selective DA sensitivity	Yes	Yes- however several studies have pointed to the selectivity of glutamate and possibly GABA
Brain regions affected	Substancia nigra pars compacta (SNpc) primarily and striatum	Globus pallidus primarily but striatum, and substancia nigra may also be involved
Mitochondrial dysfunction/ oxidative stress	Yes	Yes
L-DOPA responsive	Yes	Generally unresponsive to L-DOPA however, some motor symptoms reversed but with major side effects
Symptoms	Emotional and cognitive decline, bradykinesia, rigidity, tremors and postural instability.	Extrapyramidal syndrome
		Slowed hand movements, irritability, aggressiveness and hallucinations.
	Later stages: masked-face, forward-flexed posture, gait freezing, shuffling steps, GI issues	Later stages: Rigidity, tremor, gait disturbances (slow and clumsy movements), hypokinesia, facial muscle spasms, propensity to fall backward when pushed, less frequent resting tremor, more frequent dystonia, a "cock-walk" collectively referred to as 'manganism'

[a] Adapted and modified from (Martinez-Finley et al., 2013).

oxidative metabolism. The greatest amount of ROS is produced from electron transport chain (ETC) from mitochondria than other organelles (Martinez-Finley et al., 2013). The superoxide formed in mitochondria is converted to hydrogen peroxide and transported to other parts of cells including cytosol and nucleus. ROS species can cause damage to nucleic acids, proteins, and phospholipids both in mitochondria and cells. Mitochondrial DNA is

very sensitive to ROS as it does not have protective mechanisms and histones, leading to degeneration of mitochondria. Manganese can bind to calcium binding sites in tricarboxylic acid (TCA) cycle to increase ATP production which in turn increase ROS production. Excessive manganese can inhibit the rate of ATP production through inhibition sites in ETC (Gavin et al., 1999). Furthermore ROS can induce to have mitochondrial permeability transition (MPT). MPT dissipates electrochemical proton gradient and turns off ATP production. MPT plays a component of programmed cell death. The presence of Mn^{3+} induces more reactive oxygen stress leading to disruption of mitochondria and inducing apoptosis, a programmed cell death (Reaney and Smith, 2005).

The exact cellular and molecular mechanisms of dopaminergic neuronal cell death upon manganese exposure are still unclear. Recently, our lab demonstrated that neuronal cells after manganese exposure undergoes programmed cell death. ROS induced by manganese exposure initiates mitochondria related apoptotic process. Cytochrome C release, activation of caspase-3, and DNA fragmentation were reported in Mn induced neurotoxicity. Protein kinase C delta (PKCδ) was identified as one of the key elements in apoptotic process during manganese induced neurotoxicity (Latchoumycandane et al., 2005a, Zhang et al., 2007a). PKCδ was cleaved proteolytically by active caspase-3. The catalytic domain of PKCδ gets released from regulatory domain and activated by phosphorylation, leading to cell death. Prevention of activation of PKCδ reduced cell death during manganese exposure (Kanthasamy et al., 2003, Kitazawa et al., 2005, Latchoumycandane et al., 2005b).

Protein kinase C delta

Protein kinase C delta (PKCδ) was first identified by Gschwendt and was cloned from a rat brain cDNA library (Gschwendt et al., 1986, Gschwendt, 1999). Protein kinase C delta is located in chromosome 3 for human, chromosome 14 for mouse, and chromosome 16 for rat (Gschwendt, 1999). Based on the activation patterns, isoforms of PKCδ are classified into three groups: conventional PKC (cPKC), novel PKC (nPKC), and atypical PKC (aPKC)

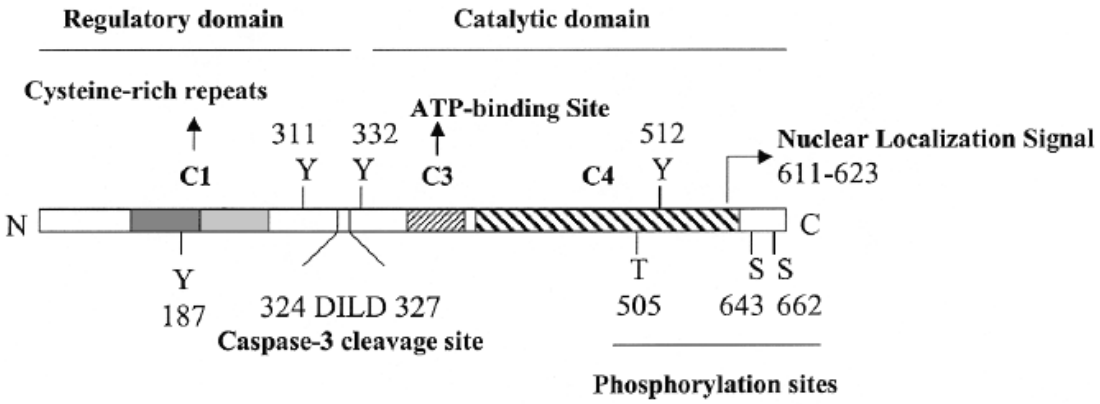

Figure 4. The structural and functional features of PKCd. Adapded and modified from (Kanthasamy et al., 2003).

(Gschwendt, 1999). PKCd belongs to nPKC and is independent on intracellular calcium concentration, while it is activated by diacylgycerol or phorbol ester. cPKC is dependent on intracellular calcium concentration, whereas aPKCs are not only independent on calcium but also not activated by phsopholipids. PKCδ has N-terminal regulator domain and C-terminal catalytic domain which are connected by variable region (Kanthasamy et al., 2003).

PKCδ is ubiquitously expressed in many tissues including brain (Leibersperger et al., 1991). Our laboratory group previously showed that PKCδ is highly expressed in substantia nigra and striatum compared to other regions in mouse brain (Zhang et al., 2007b). PKCδ can

be activated by phospholipid such as diacylglycerol and by tyrosine phosphorylation. In addition, PKCδ can be activated via cleavage of PKCδ by proteolytic activation of Casp-3.

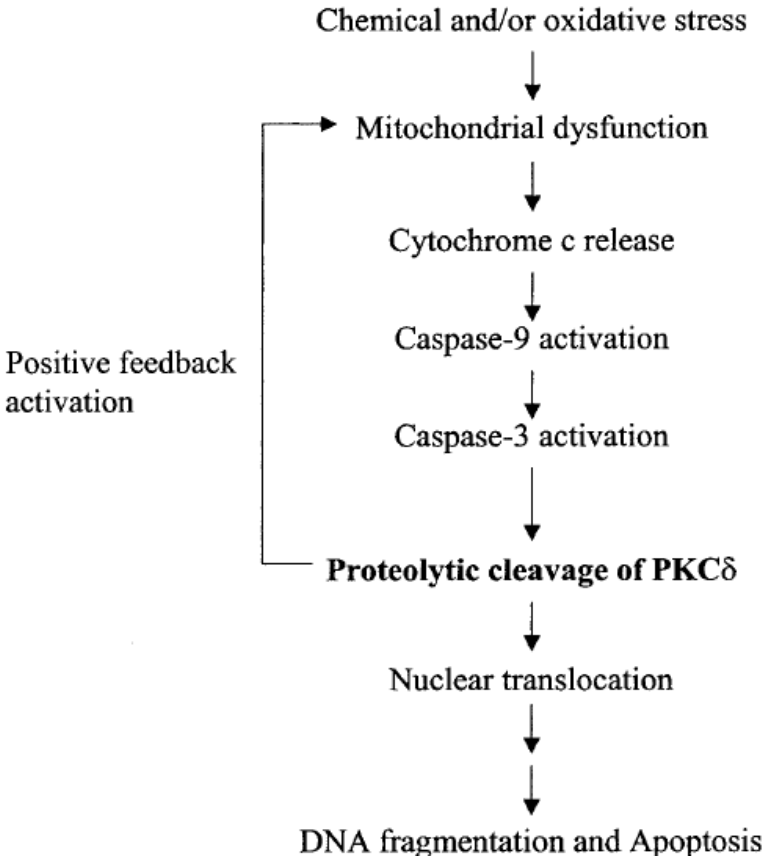

Figure 5. Shematic representation of the proapoptotic role of PKCd in oxidative stress induced apoptosis. Adapted and modified from (Kanthasamy et al., 2003)

Once activated, PKCδ translocates to the cell membrane or other subcellular organelles. Thr-505, Ser-643, and Ser-662 were identified as key phosphorylation sites in PKCδ (Kanthasamy et al., 2003). Phosphorylation of Thr/Ser in PKCδ influences the kinase activity of PKCδ (Le Good et al., 1998). PKCδ was shown to be activated in nonneuronal cell types in response to various apoptotic stimuli, including tumor necrosis factor-α (TNFα), Fas

ligand, and Interleukin-1β (Kanthasamy et al., 2003). Our group previously also showed that dopaminergic neurotoxic agents can activate PKCδ via Casp-3 mediated cleavage (Anantharam et al., 2002, Kitazawa et al., 2003, Yang et al., 2004). Active caspase-3 induces proteolytic cleavage of PKCd to yield 38 kDa of regulatory and 41 kDa of catalytic domain. Furthermore, prevention of cleavage of PKCδ with pretreatment of caspase-3 inhibitor was shown to protect dopaminergic neuronal cells (Kanthasamy et al., 2006b). PKCd is a key protein that mediates the upstream apoptotic signal to arrays of executing pathways in manganese induced neurotoxixity as well as in PD. Though PKCd offers great potential to new pharmaceutical drug candidate and good understanding of neurotoxicant induced dopaminergic neurodegeneration, much gap still needs to fill in to elucidate pathogenesis of PD and manganese induced neurodegeneration.

p73

Human p73 gene was identified in 1997 in chromosome 1p36. The *p73* gene (also known as *Trp73*) is a homolog together with p63 to p53 tumor suppressor gene which share similar domain architecture within all three gene family. Alternative splicing produces multiple mRNA transcripts which differ at C-terminus of three genes. The *p73*gene has 15 exons and two different transcription start sites. Additionally, the presence of transactivation (TA) domain adds more diversity in isoforms of the p73 transcription, as transcripts from the upstream promoter generate transactivation domain-containing isoforms of p73 (TAp73), while transcripts from the second promoter produce the N-terminal transactivation domain-truncated isoform subfamily (ΔNp73). p53 family genes including p73 play key roles in

regulators of proliferation, differentiation, cell death, and stem cell renewal. Besides, p53 and p73 are also implicated in neurodegenerative diseases such as Alzheimer's disease (Yang et al., 2002, Vousden and Lane, 2007, Killick et al., 2011, Engelmann et al., 2015). Similar to the p53 tumor suppressor, TAp73 induces apoptosis and serves as a tumor suppressor. Interestingly, ΔNp73 inhibits cellular apoptosis by directly inhibiting TAp73 activity (Nakagawa et al., 2002, Dobbelstein et al., 2005, Killick et al., 2011). Although the p53 family members, including p73, share highly similar protein architectures and cellular targets, they have different upstream signals (Rosenbluth and Pietenpol, 2008).

Both TAp73 and ΔNp73 express in various cancers including bladder, breast, colon, and neuroblastoma (Engelmann et al., 2015). Main function of TAp73 in cancer is to inhibit cell cycle progression, maintenance of genome stability and promotion of apoptotic cell death. TAp73 transcriptionally activate the targets of p53 such as p21 or GADD45 and mediates cell cycle arrest in G1 and G2/M. Genotoxic or oncogenic stress mediates TAp73 to promote mitochondria-mediated apoptosis by activating Bax, Puma, Noxa, Bad, Bik, and p53Aip1 which are targets of p53. In addition, TAp73 induces p53-independent mitochondrial apoptosis by transcriptionally activating GRAMD4. TAp73 also induces activation of pro-apoptotic signal cascade. IGFBP3 is a target of TAp73 and induces cancer cell death. Interleukin 4 (IL-4) receptor is another target of TAp73 and mediates IL4-mediated cell death. TAp73 is also implicated in autophage-mediated cell death. Both TAp73 and ΔNp73 gene products are regulated by ubiquitine-proteosomal degradation pathway. Interestingly, TAp73 responds to DNA damage and transcriptionally activate ring finger domain ubiquitin ligase, PIR2, which binds to TAp73 and ΔNp73. PIR2 stabilizes TAp73,

however, degrades ΔNp73 through ubiquitination, increasing the ratio of TAp73 over ΔNp73. Genotoxic stress also induces AP-1 transcription factor which suppress transactivation of the polyamine catabolic enzyme PAOX. Antizyme AZ1 was induced by polyamines and mediates degradation of ΔNp73.

p73 gene products participate in playing roles in development of neurons. Unlike p53

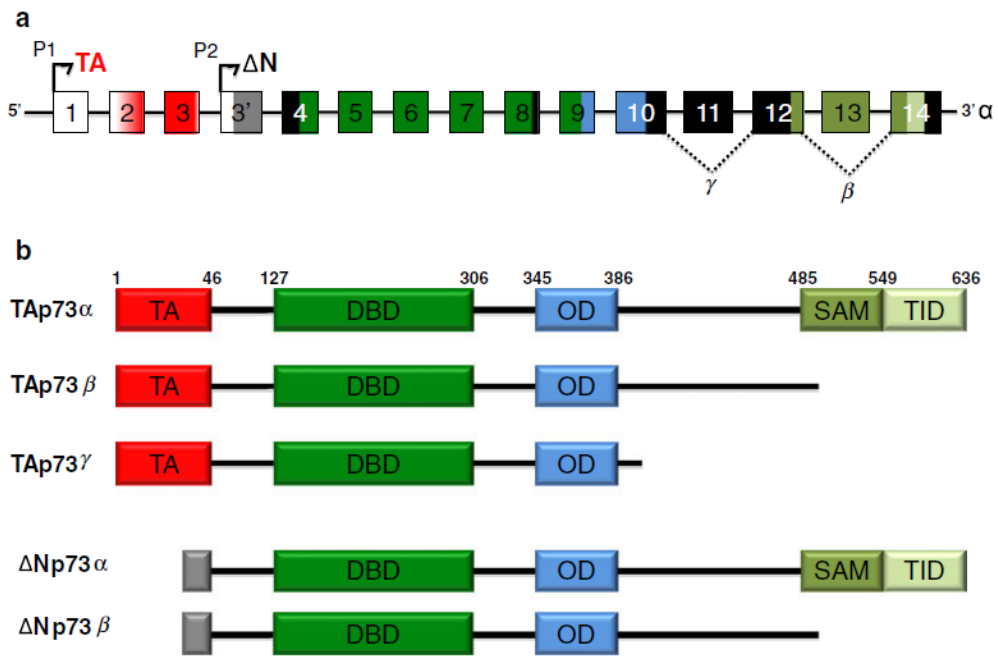

Figure 6. p73 gene structure and its splicing variants. Adapted and modified from (Killick et al., 2011).

knockout mice, p73 knockout mice do not develop tumors. However, p73 knockout mice have developmental defects in CNS including congenital hydrocephalus and hippocampa dysgenesis. P73 knockout mice also showed defect in embryonal and adult neurogenesis. The function of p73 gene related to neuronal cell survival was attributed from the loss of ΔNp73.

p73 gene was also implicated in neurodegeneration. Heterozygous $p73^{+/-}$ mice showed substantial increases in tau phosphorylation levels with filamentous aggregates

(Engelmann et al., 2015). It was proposed that ΔNp73 bind and inhibit JNK which directly targets tau, while TAp73 cannot. Thus, loss of ΔNp73 impacts on regulation of tau and contributes to Alzheimer's disease. TAp73 might have a role in regulating expression of tyrosine hydroxylase (TH), a key enzyme to produce dopamine. The promoter of TH has putative p73 binding site. TAp73 was shown to induce expression of TH in *in vitro* model (Grespi and Melino, 2012). However, these findings is needed to confirm in other *in vitro* model as well as in *in vivo* model.

Signal transducer and activator of transduction

Signal transduction and activated transducer (STAT) is a molecule that transduces many extracellular biological signals to nucleus to regulate specific gene regulation (Buitenhuis et al., 2004, Paukku and Silvennoinen, 2004, Abroun et al., 2015). STAT has seven family members and share high structural homology. Activation of STATs begins with ligands binding to their receptors in cell membrane, leading to activation of Janus-kinase (JAK) by phosphorylation. Activated Jak recruits, allows formation of hetero- or homo-dimerization of proximal STATs before activation by phosphorylation. The phosphorylated STATs translocate to nucleus where the extracellular signaling exerts fine and specific gene regulation (Paukku and Silvennoinen, 2004, Tan and Nevalainen, 2008).

The DNA-binding domain has b-sheet structure and recognize GAS element. The SH2 domain is highly conserved and play a role in dimerization with other STAT. STATs can form homodimers and heterodimers with other STAT, except STAT5. Transactivation

domain at C-terminus is poorly conserved and contains serine residues. Phosphorylation of serine residue modulate transcriptional acitivity of STATs (Decker and Kovarik, 2000).

The function of STAT1 is associated with interferon (IFN). IFN induces a homodimer

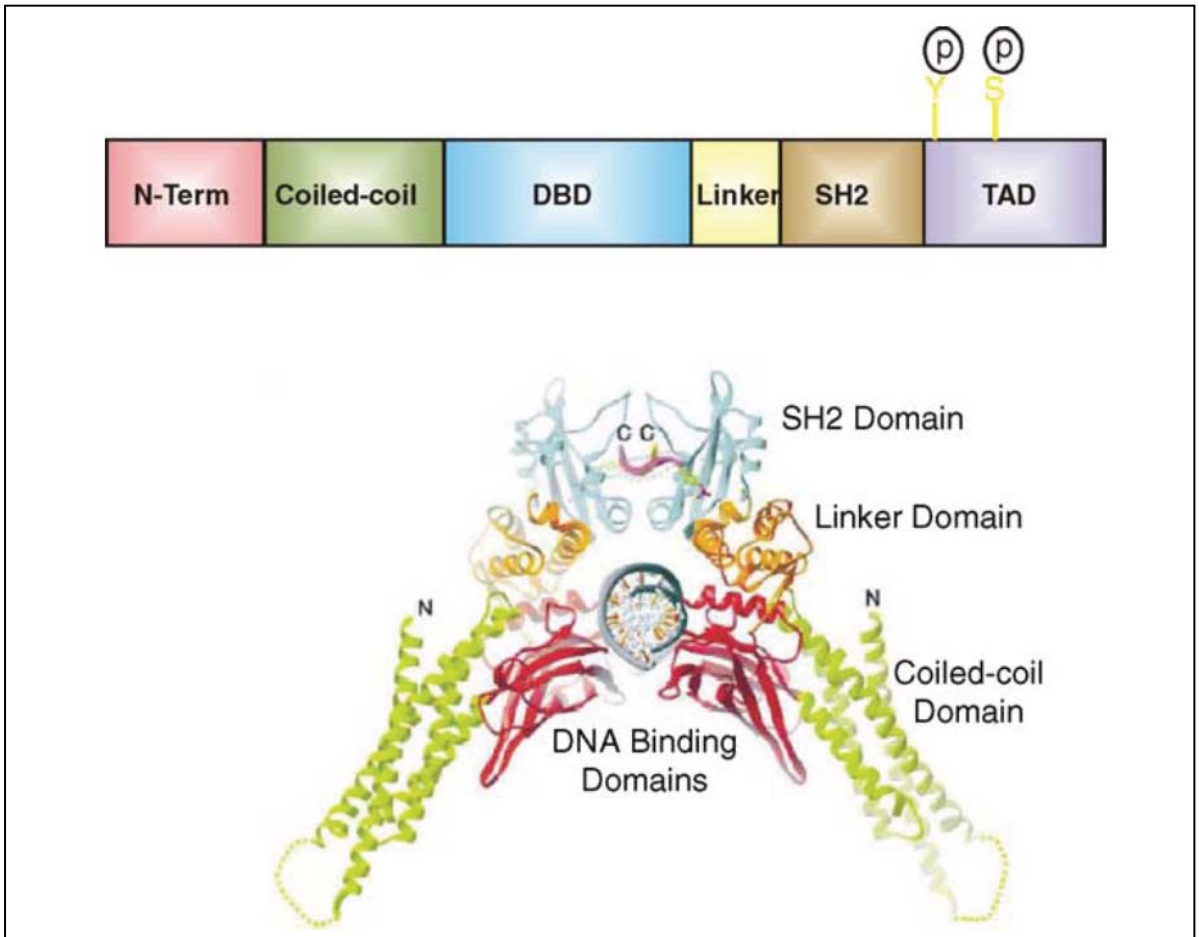

Figure 7. STAT structure. Adapded and modified from (Paukku and Silvennoinen, 2004).

of STAT1 and leading to gene expression of γ-activated sequence (GAS)-driven genes. IFNα and β induce STAT1 homodimer or STAT1-STAT2 heterodimer. STAT1 KO mice exhibit defective in innate response to viral and bacterial pathogens. STAT1 is considered

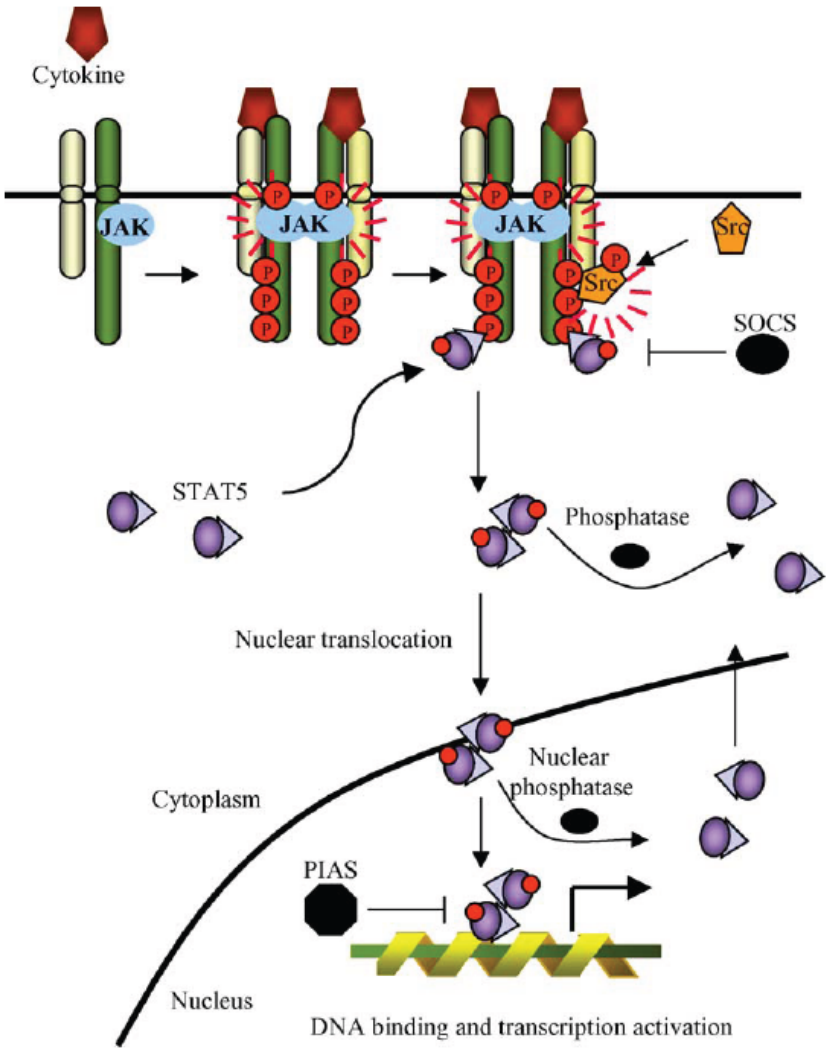

Figure 8. Schematic diagram of STAT5 pathway. Adapted and modified from (Buitenhuis et al., 2004).

as tumor suppressor as STAT1 KO mice developed tumors more readily than wild type (Durbin et al., 1996). STAT1 is also associated with proteins involved in antigen presentation (Schindler and Strehlow, 2000). STAT2 is associated with the function of IFNα and IFNβ. STAT2 KO mice were susceptible to viral infection due to impaired IFN functions. STAT3 is biquitously expressed in most tissues and its function is dependent on tissue types. STAT3 responds to IL-6 and other cytokines. STAT3 has function of proliferation in many cell types

including cancer cell line and tumors. STAT4 is expressed selectively in natural killer cells, dendritic cells and T-lymphocytes. STAT4 can be activated by IL-12 and plays a role in development of Th1 cells. STAT6 is expressed in all tissues. STAT6 is associated with Th2 differentiation and involved in adaptive immune system. STAT6 is activated by IL-13 and often functions with IL-4. STAT6 KO mice exhibits the loss of response to IL-4.

STAT5 was initially discovered as mouse mammary gland factor before renamed as STAT5 because of significant sequence homology with other member of STATs before finding of another member similar to STAT5. STAT5A and STAT5B are located on chromosome 17 in human and chromosome 11 in mouse. STAT5A and STAT5B have high sequence homology over 90% with main difference of sequence in C-terminal where transcription activation domain is located. STAT5B promoter does not have TATA or CAAT box. Instead, STAT5B contains Sp1 sites which were negatively regulated by methylation (Crispi et al., 2004). STAT5A KO mice showed loss of response to prolactine (PRL) and impairment of mammary gland development. STAT5B KO mice showed lack of response to growth hormone (GH) and impairment to growth. Decreased expression of STAT5B has also been shown to compromise cellular protection against reactive oxygen species (ROS) in peripheral system (Du et al.). Although STAT5 A/B KO mice is viable,

Table 3. Characteristics of STAT5 [a]

Member	Chromosomal location	Isoform	Gene size (bp)	mRNA size (bp)	Amino acid	MW (KDa)
STAT5	17q11.2	a	24,397	4,314	794	
		b	77,230	5,171	787	90

[a] Adapted and modified from (Abroun et al., 2015).

STAT5 A/B KO mice showed defective mammary gland and reduced body mass with defective T-cell proliferation (Liu et al., 1997, Teglund et al., 1998, Miyoshi et al., 2001).

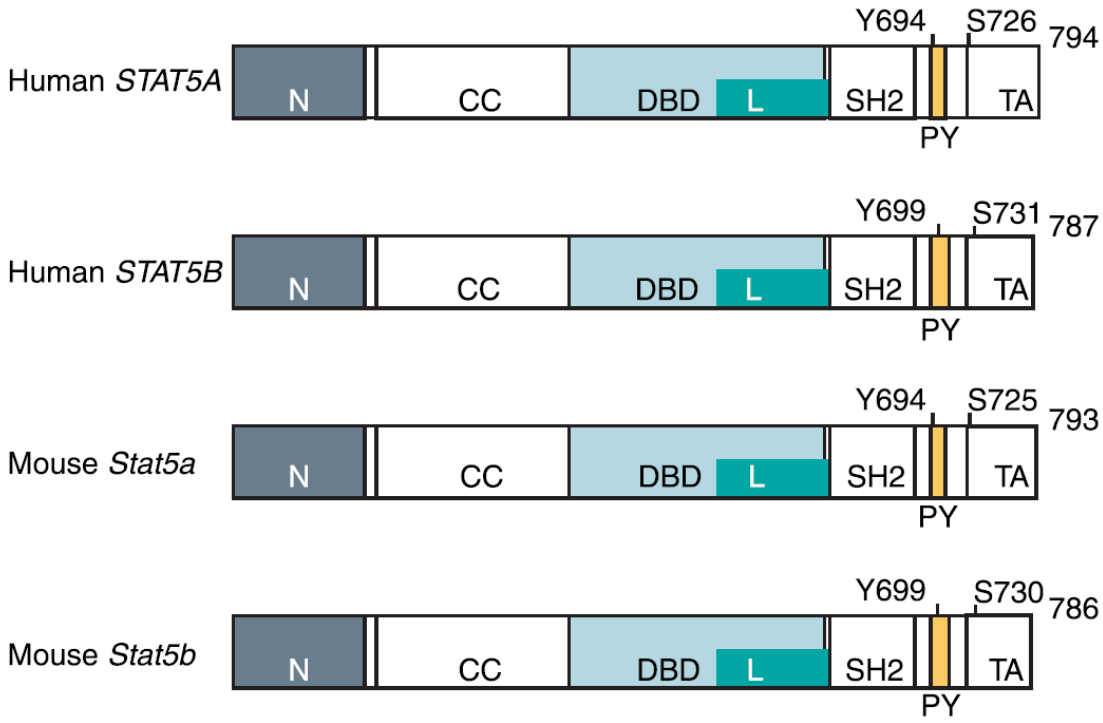

Figure 9. Structural functional domain of STAT5. Adapted and modified from (Tan and Nevalainen, 2008).

Though STAT5A and STAT5B share their target genes, they have distinctive roles in various conditions. They have non-redundant target gene, different profiles of interactions with other co-factors, and different cell types-specific expression profiles (Tan and Nevalainen, 2008, Kanai et al., 2014). While many tissues have equal expressions of STAT5A and STAT5B, some tissues have more preferential expression of either form. STAT5A is more prevalent in mammary gland, while STAT5B is more abundant in liver and muscle. Bain showed more expression of STAT5B (Liu et al., 1995).

STAT5 can be activated by several ways. Cytokine can bind to its receptor and trigger activation of STAT5B via JAKs. Known cytokines to activate STAT5 are listed: LIF,

Leptin, IL-3, IL-4, IL-7, IL-10, IL-13, IL-21, IL-22, IL-27, GM-CSF, Epo, IFNα, IFNβ, EGF and PDGF (http://www.cellsignal.com/common/content/content.jsp?id=science-tables-jak-STAT). In addition to cytokine receptors which rely on JAK to activate STAT due to the lack

Table 4. Targets of human STAT5 [a]

Gene	Location	Gene	Location	Gene	Location	Gene	Location
ANGPTL4	19p13.3	*CSN2*	4q21.1	*IL6ST*	5q11	*PRF1*	10q22
BCL2	18q21.3	*EGFR*	7p12	*MET*	7q31	*RARA*	17q21
BCL2L1	20q11.21	*ESR1*	6q25.1	*MUC1*	1q21	*RNMT*	18p11.22
BCL6	3q27	*ESR2*	14q	*OSM*	22q12.2	*SEC6L1*	5p15.33
CCND1	11q13	*IFNG*	12q14	*PAX5*	9p13	*TIMP3*	22q12.3
CCND2	12p13	*IGF1*	12q22-23	*PBF*	8p21.1	*TNF*	6p21.3
CCND3	6p21	*IL2RA*	10p15-	*PIM1*	6p21.2	*TNFRSF5*	20q12
CEL	9q34.3	*IL6*	7p21	*PPARG*	3p25	*TRIP15*	15q21.2

[a] Adapted and modified from (Abroun et al., 2015).

of cytoplasmic kinase domain, some tumors constitutively activates tyrosine kinases including JAK1, JAK2 and TYK2 (Abroun et al., 2015). Receptor tyrosine kinases such as Fyn were shown to activate STAT5 as well (Pullen et al., 2012). STAT can also be negatively regulated. Phospho tyrosine phosphatase 1B was shown to de-activate STAT5 (Aoki and Matsuda, 2000). Suppressors of cytokine signaling were shown to negatively regulate STAT (Zhang et al., 1999). It was reported that STAT5 can be negatively regulated via proteasomal degradation pathway (Wang et al., 2000). However, recent follow-up studies

showed that there was mishap during experiments (Ramos et al., 2007). In addition, transcription factors such as NF-kB was shown to negatively regulate the function of STAT5 (Geymayer and Doppler, 2000). Moreover, miRNA was also shown to involve regulation of STAT5. miR200a was shown to negatively regulate STAT5 expression during pregnancy (Williams et al., 2012a).

As described above, STAT5 has functions related with mammary gland and growth hormone related response. The patients with missense mutation in STAT5B exhibited retardation in growth and severe immunodeficiency (Kanai et al., 2012). STAT5 is implicated in multiple cancers (Tan and Nevalainen, 2008, Wagner and Schmidt, 2011). Often times STAT5 pathway was constitutively activated. STAT5B was reported to have non-canonical functions. STAT5B was shown to be implicated with structural function in Golgi apparatus and endoplasmic reticulum (Sehgal, 2013). Furthermore, STAT5 was shown to play a role in mitochondria (Meier and Larner, 2014). STAT5B was translocated in mitochondria after IL-3 stimulation (Chueh et al., 2010) and associated with pyruvate dehydrogenase complex-E2 (Chueh et al., 2011). STAT5 was shown to bind to D-loop in mitochondrial genome (Chueh et al., 2010).

Overarching hypotheses and rationale for these studies

As described above chronic exposure to low level of Mn induces neurodegeneration and show Parkinson's like symptoms. Recent research extensively studied and identified several key molecules such as PKCd, Caspase-3 and p53 (Latchoumycandane et al., 2005b, Zhang et al., 2007b, Jin et al., 2011, Uchida et al., 2012, Jin et al., 2014, Wan et al., 2014, Ma

et al., 2015). Nonetheless, the exact molecular and cellular mechanisms underlying Mn-induced neurotoxicity have not been elucidated. Hence, identifying the key molecules in Mn-induced neurotoxicity is essentially required. The present studies seek to identify the key molecules and understand molecular mechanisms of the molecules in Mn-indueced neurotoxicity.

CHAPTER II: *p73* GENE IN DOPAMINERGIC NEURONS IS HIGHLY SUSCEPTIBLE TO MANGANESE NEUROTOXICITY

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Abstract

Chronic exposure to elevated levels of manganese (Mn) has been linked to a Parkinsonian-like movement disorder, resulting from dysfunction of the extrapyramidal motor system within the basal ganglia. However, the exact cellular and molecular mechanisms of Mn-induced neurotoxicity remain elusive. We treated C57 black mice with 30 mg/kg Mn via oral gavage for 30 days. Interestingly, we found a significant downregulation of the truncated isoform of p73 protein at N-terminus from nigral tissues of Mn-exposed mice. To further determine the functional role of Mn-induced p73 downregulation in Mn neurotoxicity, we examined the interrelationship between the effect of Mn on*p73*gene expression and apoptotic cell death in an N27 dopaminergic neuronal model. Consistent with our animal study, a 300μMMn treatment significantly suppressed p73mRNA expression in N27dopaminergic cells. We further determined that protein levels of theΔNp73 isoform with truncated transactivation domain at N-terminus was also reduced in Mn-treated N27 cells and primary striatal cultures. Furthermore, overexpression of ΔNp73 confers modest cellular protection against Mn-induced neurotoxicity. Taken together, our results demonstrate that Mn exposure downregulates *p73* gene expression resulting in enhanced susceptibility to apoptotic cell death. Thus, further characterization of the cellular mechanism underlying *p73* gene downregulation will improve our understanding of the molecular underpinnings of Mn neurotoxicity.

Keywords: Manganese, p73, neurotoxicity, ΔNp73

1. Introduction

Neurodegenerative diseases are becoming more prevalent due to aging populations, genetic alterations, and exposure to an increasing array of environmental toxicants such as pesticides, neurotoxic metals and industrial byproducts (Caudle et al., 2012, Kanthasamy et al., 2012, Goldman, 2014, White et al., 2015). The transition metal manganese (Mn) is an essential trace nutrient that not only serves as a co-factor for a variety of enzymes including superoxide dismutase 2 and glutamine synthetase, but is also important for various normal physiological functions including bone formation, fat and carbohydrate metabolism, as well as the regulation of blood sugar, cellular energy, the immune system and calcium absorption (Aschner et al., 2009). Mn has been used widely in various industries for more than two centuries. The primary routes of Mn exposure are inhalation in occupational settings and dietary consumption in general. Airborne Mn particles from various chemicals including methylcyclopentadienyl manganese tricarbonyl (MMT) and fungicide are another important source of public exposure (Bowman et al., 2011, Caudle et al., 2012). Mn overexposure causes a neurodegenerative disorder known as manganism. Many clinical symptoms of manganism are also seen in PD patients, including gait dysfunction, postural instability, bradykinesia, rigidity and a mask-like face expression (Guilarte, 2011). Pathologically, both manganism and PD show dopaminergic neuronal cell death in the substantia nigra (SN) and striatum (STR). But unlike PD, manganism is unresponsive to L-dopa therapy and develops psychological abnormalities in its early phase.

The *p73* gene (also known as *Trp73*) belongs to the p53 tumor suppressor gene family, and like other family members, codes for multiple mRNA transcripts (Rosenbluth and

Pietenpol, 2008). Besides playing an important role in regulating differentiation, cell death, and neural stem cells, p73 is also implicated in neurodegenerative diseases such as Alzheimer's disease (Yang et al., 2002, Killick et al., 2011, Engelmann et al., 2015).The *p73*gene has 15 exons and two different transcription start sites. The C-terminal domain of p73 is alternatively spliced generating a variety of isoforms. Transcripts from the upstream promoter generate transactivation domain-containing isoforms of p73 (TAp73), while transcripts from the second promoter produce the N-terminal transactivation domain-truncated isoform subfamily (ΔNp73). Similar to the p53 tumor suppressor, TAp73 induces apoptosis and serves as a tumor suppressor. Interestingly, ΔNp73 inhibits cellular apoptosis by directly inhibiting TAp73 activity (Nakagawa et al., 2002). The p53 family members, including p73, share highly similar protein architectures and cellular targets, but they have different upstream signals (Rosenbluth and Pietenpol, 2008).

Manganese-induced neurotoxicity has been studied extensively, and several key players, such as protein kinase C delta (PKCδ) (Latchoumycandane et al., 2005, Jin et al., 2011),SLC30A10 (Leyva-Illades et al., 2014, Chen et al., 2015), ATM-p53 (Tidball et al., 2015), and others (Horning et al., 2015) have been identified. However, the cellular and molecular mechanisms underlying Mn-induced neurotoxicity still have not been delineated. Here, we report that ΔNp73 expression was downregulated during Mn exposure in N27 dopaminergic neuronal cells, mouse primary striatal cells, and in the mouse SN. Through functional studies, we identified that ΔNp73 downregulation makes neuronal cells more susceptible to Mn Neurotoxicity. Overexpression of ΔNp73 protects N27 dopaminergic

neuronal cells from Mn-induced neurotoxicity. These observations provide new insights into the mechanisms underlying Mn-induced dopaminergic neurotoxicity.

2. Materials and Methods

2.1 Chemicals

Manganese chloride ($MnCl_2$, 99%) was purchased from Sigma-Aldrich (St. Louis, MO). Antibodies against Bcl-xL, Mcl-1, and TAp73were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody against ΔNp73 was purchased from Imagnex (San Diego, CA).Bradford protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA). Mouse and rabbit monoclonal antibodies against β-Actin were purchased from Sigma-Aldrich. The anti-mouse and anti-rabbit secondary antibodies (Alexa Fluor 680 conjugated anti-mouse IgG and IRDye800 conjugated anti-rabbit IgG) were purchased from Life Technologies (Grand Island, NY) and Rockland (Gilbertsville, PA), respectively. RPMI1640, fetal bovine serum, L-glutamine, penicillin, and streptomycin were purchased from Life Technologies. We purchased the tetrapeptide caspase-3 inhibitor zDEVD-fmk from ApexBio (Houston, TX).

2.2 Animal studies

Six- to eight-week-old male C57BL/6J mice were housed at RT under a 12 h light cycle with water and food provided *ad libitum*. Mn (10 or 30mg/kg) was orally administered once daily for 30 days. Animals were cared for in accordance with Institutional Animal Care and Use committee guidelines. After the treatment period, striatal tissues were collected for apoptosis PCR array analysis or for measuring the expression of proteins of interest.

2.3 Cell cultures

A rat-derived dopaminergic neuronal cell line, N27, was kindly gifted from Dr. Kedar N. Prasad (University of Colorado Health Sciences Center, Denver, CO). N27 cells were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 units of penicillin, and 50 µg/ml of streptomycin in a humidified incubator with 5% CO_2 at 37°C as described previously (Jin et al., 2011).

2.4 Primary striatal cultures and treatment

Primary striatal neuronal cultures were prepared from the ganglionic eminences of gestational 14- to 15-day-old mouse embryos as previously described (Jin et al., 2011). Briefly, after extracting the embryonic brain, the embryonic cerebral cortex was folded away to expose ganglionic eminences followed by dissection of the STR, which were then washed in Dulbecco's modified eagle media (DMEM) and dissociated via trypsinization. Dissociated cells were seeded into poly-D-lysine-coated plates and maintained in Neurobasal medium supplemented with B-27 supplements, 500mM L-glutamine, 100 IU/ml penicillin, and 100µg/ml streptomycin in a humidified incubator with 5% CO_2 at 37°C. Four- to six-day-old primary striatal neuronal cells were exposed to various concentrations of Mn (100-500 µM) for 24 h and then subjected to Western blot analysis.

2.5 Plasmid constructs

The expression plasmid for the human p73 alpha isoform (HA-p73alpha-pcDNA3) was obtained from Addgene (ID 22102) as described (Jost et al., 1997). To generate the expression vector for the N-terminal transactivation domain-truncated p73isoform, the appropriate cDNA fragment was PCR-generated fromHA-p73alpha-pcDNA3 using a

forward primer, 5'-CAGATTACGCTAGCTGCTGTACGTCGGTGACCCCGCACGGCACCTCGCCACGGCCCAGTTCAATCTGCTGAGCAGCACC-3', and the SP6 reverse primer. The restriction enzymes of NheI and Xho I were used to generate the5' overhang cleavage site, which was used for the ligation reaction with pcDNA3 vector. The resulting clone for ΔNp73 was confirmed by sequencing.

2.6 Transfections

N27 cells were transfected with ΔNp73DNA construct through electroporation. The Amaxa Nucleofector instrument was used according to the manufacturer's protocol. Five million cells were transfected with 10μg of plasmids using the A-23 program. Transfected cells were seeded in either T-75 flasks or 96-well plates as desired for 24h before treatment (Zhang et al., 2007b).

2.7 Cell viability assays

Colorimetric MTS assay was performed to measure cell viability using the Cell Titer 96 Aqueous One Solution Proliferation Assay kit from Promega as described previously (Jin et al., 2014a). Briefly, cells were seeded in 96-well plates before exposure to Mn. At the end of treatment, 20% MTS reagent was added to each well. The MTS tetrazolium compound is bio-reduced in living cells to its colored formazan products. After incubating for up to one hour, absorbance at 490nm was measured using a SpectraMax spectrophotometric microplate reader (Molecular Devices).

2.8 Quantitative real-time RT-PCR

After treatment, total RNA was extracted from cells using the Absolutely RNA Miniprep kit and converted to cDNA using the High Capacity cDNA Synthesis kit (Life Technologies). SYBR Green quantitative PCR was performed according to the manufacturer's protocol. Forward primer (5'-ACTGCAAGTCCCGTAAGCAG-3') and reverse primer (5'-CATGAGCGACTTCCCTTCAG-3') were used for detecting p73 mRNA. Validated primers for 18S rRNA (Qiagen, #PPR57734E) were used as the housekeeping gene controls. The threshold cycle (C_t) was calculated from the instrument software, and fold change in gene expression was calculated using the $\Delta\Delta C_t$ method.

2.9 Behavioral measurements

Spontaneous exploratory movements of mice were measured using a computer-controlled open-field apparatus (VersaMax animal activity monitor, model RXYZCM-16, Accuscan, Columbus, OH) as described previously (Zhang et al., 2007a).The transparent activity chamber was 20 × 20 × 30.5 cm and was covered with a transparent ventilated lid. Data were collected and analyzed by a VersaMax analyzer (model CDA-8, AccuScan). Following a 2-min adaptation period, each mouse was given 10 min to explore the open-field monitor while horizontal and vertical movements were recorded automatically *via* an array of infra-red beams.

2.10 Western blotting

Cells were lysed in lysis buffer (1% Triton X-100, 1mM EDTA, 100mM NaCl, 1mM EGTA, 1mM NaF, 20mM $Na_4P_2O_7$, 2mM Na_3VO_4, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 50mM Tris-Cl, pH7.4). Brain homogenates were prepared as described

previously (Jin et al., 2011).Western blotting was performed as described previously (Jin et al., 2014b). Briefly, the samples containing equal amounts of proteins were fractionated through a 10-15% SDS-PAGE gel and transferred onto a nitrocellulose membrane. Primary antibodies against specific proteins were incubated with the membrane. After thoroughly rinsing in PBS supplemented with 0.1% Tween-20, the membrane was incubated with Alexa Fluor 680 goat anti-mouse, Alexa Fluor 680 donkey anti-goat, or IRDye 800 donkey anti-rabbit secondary antibodies. For the loading control, β-Actin antibody was used. Immunoblot imaging was performed with an Odyssey Infrared Imaging system (LI-COR, Lincoln, NE, USA). ImageJ software (National Institutes of Health, Bethesda MD, USA) was used to quantify Western blot bands.

2.11 Statistical analysis

Data were analyzed using Prism 4.0 (GraphPad Software, San Diego, CA). Tukey's multiple comparison test was used after ANOVA for comparing differences between multiple groups. Paired student's *t*-test was used when two groups were being compared. Differences were considered statistically significant for p-values <0.05. Data are represented as the mean ± S.E.M. of at least two separate experiments performed at least in triplicate.

3. Results

3.1 Manganese exposure alters apoptosis-related gene expression in mouse substantia nigra

Chronic Mn exposure induces a neurotoxic response in the nigrostriatal system of the basal ganglia (Latchoumycandane et al., 2005, Zhang et al., 2007b, Farina et al., 2013,

Martinez-Finley et al., 2013). The tumor suppressor *p73* gene encodes two major groups of p73 isoforms with opposing cellular functions. The full-length p73 protein (TAp73) is known to induce apoptosis, while its truncated isoforms (ΔNp73) lacking the N-terminal domain exert anti-apoptotic activity (Muller et al., 2005, Ozaki et al., 2005, Killick et al., 2011). To investigate whether Mn exposure has any effect on the expression of *p73* genes *in vivo*,C57BL mice were orally treated with 30 mg/kg/day of Mn for 30 days and then their nigral tissues were subjected to biochemical analysis. To perform isoform specific gene expression analyses of *p73* gene products, we used two specific antibodies directed against full-length and truncated p73 proteins, respectively. Interestingly, expression of ΔNp73 in Mn-treated mice was reduced by more than 50% when compared to control mice (Fig. 1C-D). Full length *p73* protein expression was not changed (data not shown). Behavioral performance testing also confirmed that 30 mg/kg Mn treatment caused significant motor deficits (Fig. 1A-B). These findings in an animal model indicate that Mn exposure induced downregulation of ΔNp73 expression.

3.2 Manganese exposure downregulates ΔNp73 expression in N27 dopaminergic cells and mouse primary striatal cultures

To further characterize the downregulation of *p73* gene expression and its function during Mn exposure, we used the *in vitro* N27 dopaminergic neuronal cell system. N27 cells were exposed to 300 µM Mn. The quantitative RT-PCR analysis was performed for *p73* gene expression (Fig. 2A). We found ~60% reduction of *p73* gene expression relative to control cells. Since *p73* gene function is not known in Mn neurotoxicity, we characterized the role of

p73 using cell culture models. The expression of *p73* was analyzed by Western blot assay using specific antibody directed against truncated *p73* proteins. The truncated isoform of *p73* gene product was downregulated upon exposure to 300 µM in a time-dependent manner with ~60% reduction relative to control cells at 24 h treatment (Fig. 2B-C), which validated the results from our *in vivo* model and also showed that Mn exposure affected dopaminergic neurons and downregulated expression of ΔNp73 in dopaminergic neurons. N27 cells treated with 300 µM Mn for 12hdid not trigger cell death, indicating downregulation of ΔNp73 takes place prior to cell death. To further validate the Mn-induced downregulation of ΔNp73, we showed that in mouse primary striatal cultures exposed to increasing concentrations of Mn (100-500 µM) for 24 h, the expression of ΔNp73 was dose-dependently repressed by Mn(Fig. 3A-B), demonstrating that Mn downregulates *p73* in an isoform-specific manner.

3.3 Downregulation of ΔNp73is not dependent on manganese-induced caspase-3 activation

Previous evidence indicates that manganese-induced neurotoxicity involves activation of caspase-3 signaling (Anantharam et al., 2004, Latchoumycandane et al., 2005). Moreover, caspase-3 has been shown to cleave p73 (Sayan et al., 2008).To investigate the possibility that activated caspase-3 contributes to the reduction of ΔNp73 protein levels by inducing proteolytic cleavage of ΔNp73, we used the cell-permeable, irreversible caspase-3 inhibitor zDEVD-fmk to selectively inhibit caspase-3 activation (Anantharam et al., 2004). Pre-treatment of naïve N27 cells with zDEVD-fmk did not alter the expression level of ΔNp73. N27 dopaminergic cells were pre-exposed to either 20 or 50µM of zDEVD-fmk for 1 h

before exposure to 300μM of Mn. Interestingly, the Mn-induced downregulation of ΔNp73 was unaffected by the presence of zDEVD-fmk (Fig. 4A-B), indicating that caspase-dependent proteolytic cleavage is unlikely to be involved in the suppression of ΔNp73.

3.4 Downregulation of ΔNp73 signaling sensitizes neurons to manganese-induced neurotoxicity

Mn exposure induces mitochondria-mediated apoptosis in neurons, which is partially facilitated by p53 (Wan et al., 2014),and ΔNp73 antagonizes the functional p53 by regulating the expression of anti-apoptotic molecules such as Bcl-xL and MCl-1 (Basu and Haldar, 1998, Grob et al., 2001, Melino et al., 2002, Huttinger-Kirchhof et al., 2006, Killick et al., 2011, Conforti et al., 2012). We therefore investigated whether downregulation of ΔNp73 affects Mn-induced dopaminergic neuronal cell death by altering the expression of key anti-apoptotic proteins, such as Bcl-xL and Mcl-1. Interestingly, we found that expression of the anti-apoptotic proteins Bcl-xL (Fig. 5A-B) and Mcl-1 (Fig. 5C-D) was remarkably reduced by ~40-50% in N27 cells treated with 300 μM Mn. These results indicate that reduced ΔNp73 levels may play an important role in Mn neurotoxicity. To further investigate the functional relevance of ΔNp73 downregulation, we overexpressed ΔNp73 in N27 cells and then exposed the cells to increasing concentrations of Mn for 24 h. ΔNp73 was overexpressed more than two-fold compared to vector control (Fig. 6A-B). Quantification of cell viability using MTS assay revealed that overexpression of ΔNp73 modestly inhibited the Mn-induced loss of cell viability during exposure to 100 and 200 μM Mn but unable to overcome the toxic effect of 300 μM Mn (Fig. 6C). Taken together, these data suggest that

ΔNp73 suppression sensitizes dopaminergic cells to Mn-induced cell death and that the suppression of the anti-apoptotic ΔNp73 protein may contribute to Mn-induced neurotoxicity.

4. Discussion

In the present study, we provide evidence that the tumor suppressor gene *p73* is highly susceptible to Mn-induced neurotoxicity in the nigrostriatal system. To our knowledge, this is the first evidence that Mn exerts its neurotoxic effects by suppressing expression of the anti-apoptotic ΔNp73 protein, thereby providing novel mechanistic insights into the molecular events underlying Mn neurotoxicity.

Although several molecules have been identified as key mediators of Mn neurotoxicity leading to apoptosis, including the pro-apoptotic kinase PKCδ (Latchoumycandane et al., 2005, Zhang et al., 2007b), caspase-12, caspase-3 (Chun et al., 2001, Wang et al., 2015), and p53 (Yang et al., 2007, Ma et al., 2015), the exact molecular and cellular mechanisms of how chronic Mn exposure induces dopaminergic neuronal cell death have not been fully characterized. The p73 protein has been shown to participate in a wide variety of cellular events, including cell death, self-renewal and cell cycle regulation (Killick et al., 2011, Di et al., 2013, Fatt et al., 2014, Engelmann et al., 2015). The p73protein has several isoforms characterized by alternative transcription at the N-terminus and alternative splicing at the C-terminus. Interestingly, TAp73isoforms having the N-terminal transactivation domain induce apoptosis, whereas ΔNp73isoforms lacking the transactivation domainantly agonize TAp73 functions, thereby favoring cell survival. We examined p73 protein expression in an *in vivo* animal model exposed to 30 mg/kg for 30 days. Mn exposure

reduced total distance of movement compared to controls, showing Mn exposure induced SN degeneration (Fig. 1A-B). We also report that downregulation of the truncated p73 isoform, ΔNp73, may play an important role in regulating neuronal cell survival during Mn exposure (Fig. 1C-D).

Characterizing the isoform function of p73 may provide a better understanding of how Mn overexposure induces dopaminergic neuronal cell death. Our quantitative RT-PCR results revealed that Mn exposure downregulates *p73* gene expression in N27 dopaminergic neuronal cells (Fig. 2A). The dose of 300 μM Mn in N27 cells is lower than 0.5-1 mM Mn used to induce toxic effects on mouse hippocampal neurons or human neuroblastoma cells (Tamm et al., 2008, Yoon et al., 2011). The concentration of Mn can reach up to 350 μM in certain brain regions (Ingersoll et al., 1999). Adult mice exposed to 30 mg/kg for 8 weeks showed the symptoms of Mn-induced toxicological effects (Moreno et al., 2009). Thus Mn concentrations used in our study were within the relevant toxicological range. The downregulation induced by 300 μM Mn occurred over 12 h in N27 cells, indicating that ΔNp73 downregulation occurred during early stages of the cell death process. Our results from primary striatal neuronal cells exposed to different doses of Mn revealed that Mn downregulated the expression of ΔNp73 in a dose-dependent manner. Caspase-3 was shown to cleave full-length TAp73 under apoptotic conditions, releasing a 45 kDa fragment (Sayan et al., 2008). Our inhibition of caspase-3 failed to recover ΔNp73 suppression. Moreover, we did not observe a ~40 kDa cleaved product of ΔNp73. These data indicate that ΔNp73 suppression involves caspase-3-independent mechanisms. Interestingly, p73 undergoes proteasomal degradation (Bernassola et al., 2004, Asher et al., 2005, Jung et al., 2011), but

further research is needed to reveal whether p73 degradation plays a role in Mn-induced ΔNp73 repression.

In addition to caspase-mediated proteolysis and proteasomal degradation, transcriptional suppression of the *p73* gene is another important mechanism to negatively alter p73 activity (Chen et al., 2001, Putzer et al., 2003). Runt-related transcription factor 2 and the transcription factor repressor ZEB bind to p73 promoter and transcriptionally suppress p73 expression (Fontemaggi et al., 2001, Bui et al., 2009, Ozaki et al., 2014). Additionally, epigenetic mechanisms, such as DNA hypermethylation, have been suggested to account for ΔNp73 transcriptional repression (Lai et al., 2014). Mn exposure induces DNA damage via oxidative stress (Oikawa et al., 2006, Stephenson et al., 2013) and upregulates p53 (Wan et al., 2014). Furthermore, genotoxic stress has been shown to selectively degrade ΔNp73 via the antizyme AZ1 (Dulloo et al., 2010). It is plausible that DNA damage induced by Mn exposure via oxidative stress may reduce ΔNp73 and antagonize TAp73 as well as p53. Altered mRNA stability, protein stability and protein degradation mechanisms may also contribute Mn-induced ΔNp73 downregulation. Future studies will focus on providing further evidence and mechanisms for Mn-induced epigenetic transcriptional repression of the *p73* gene.

Bax is one of the transcriptional targets of the *p73* gene (Melino et al., 2004, Graupner et al., 2011). Consistent with a previous report (Gonzalez et al., 2008), our results indicate that Mn exposure upregulated Bax (data not shown). Bcl-xL and Mcl-1 were reported to suppress activation of Bax and Bak, respectively (Sedlak et al., 1995, Finucane et al., 1999). Downregulation of Bcl-xL and Mcl-1 during Mn exposure (Fig. 5) indicates that

downregulation of ΔNp73 may play a role in fine-tuning the activity of p73. When we overexpressed ΔNp73 in N27 dopaminergic neurons, we observed a 2-fold increase in ΔNp73 expression compared to vector-expressing N27 cells. After 24 h-post transfection, ΔNp73 overexpression modestly inhibited against Mn-induced loss of viable cells (Fig. 6C) at low doses of 100 and 200 μM Mn, but not against the 300 μM dose. Considering that chronic exposure to low doses of Mn leads to neurodegeneration in the long-term, the modest protective effects against low Mn doses offered by ΔNp73 overexpression may play an important role. Further findings from an animal model of Mn neurotoxicity also supports that ΔNp73 expression was downregulated in Mn-treated mice relative to controls, providing *in vivo* evidence that ΔNp73 plays an important role in Mn-induced neurotoxicity. Since Mn exposure downregulates ΔNp73 in an isoform-specific manner, this gene may have a potential value in the development of a biomarker for Mn exposure.

In conclusion, our results demonstrate that ΔNp73downregulation significantly contributed to Mn neurotoxicity. These observations provide new insights into the mechanisms underlying Mn-induced dopaminergic neurotoxicity. Further characterization of the molecular mechanisms underlying ΔNp73downregulation will provide a better understanding of Mn-induced neurotoxicity.

Conflict of interest

The authors declare no conflicts of interest.

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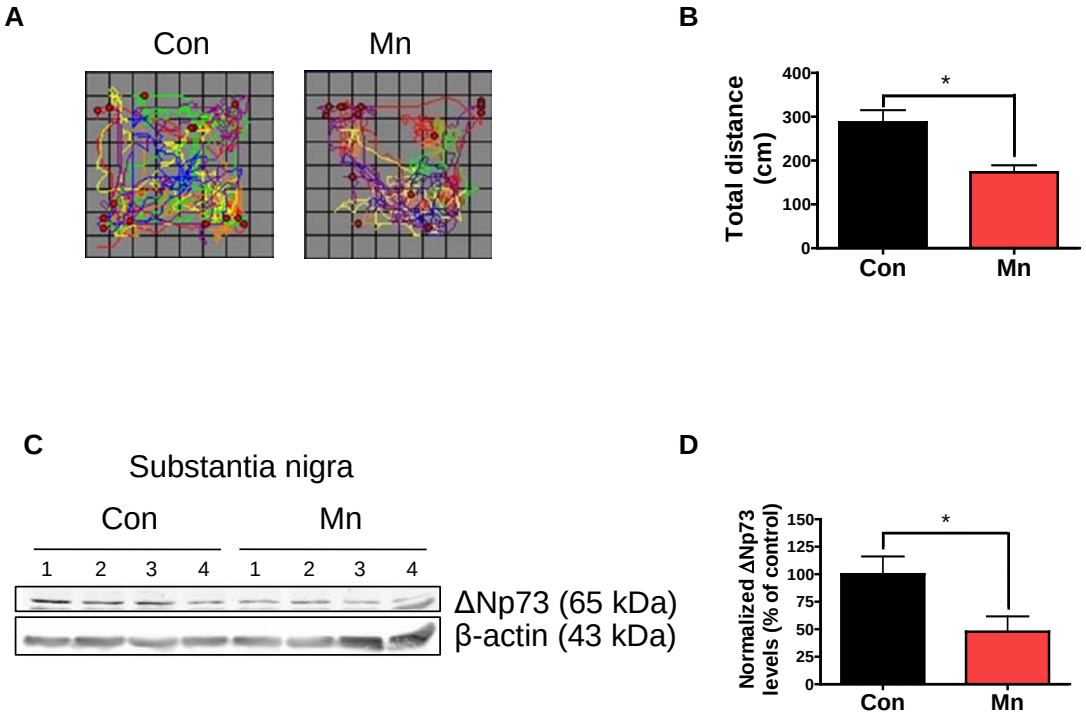


Fig 1: Oral Mn administration downregulates ΔNp73 expression in the C57BL mouse model. Male C57BL mice were orally administered 30 mg/kg of Mn for 30 days, and vehicle control animals were administered de-ionized water. After treatments, locomotor activity was measured using an automated VersaMax locomotor activity monitor. Representative activity maps of open-field movements of Mn-treated mice and control mice revealed Mn-induced locomotor deficits (A). Mn treatment also reduced total distance traveled (cm) during a 10 min test of locomotor activity(B). Western blot assays were performed on substantia nigral lysates to measure the expression of ΔNp73 (C). Densitometric analysis was also performed (D). Data are expressed as percent of control and represented as mean ± S.E.M. from four

animals per group. Asterisk (n=6-9, *, $p < 0.05$) indicates significant differences between treatment and control groups.

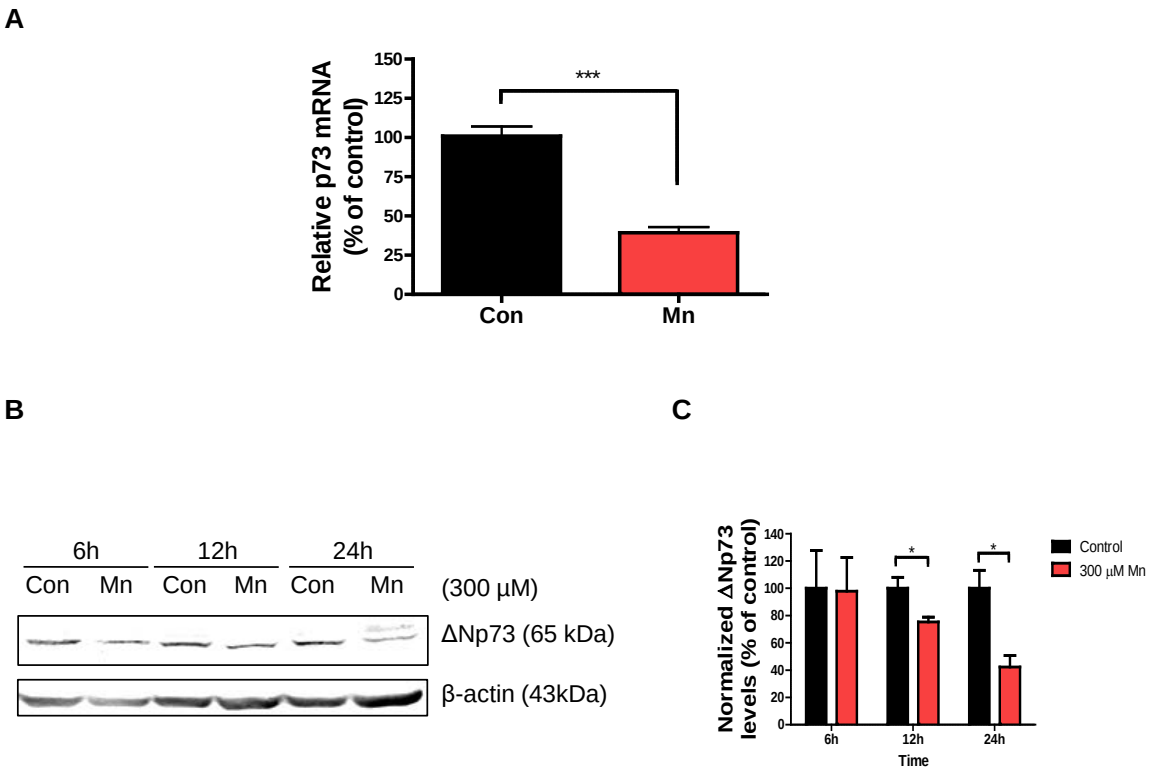
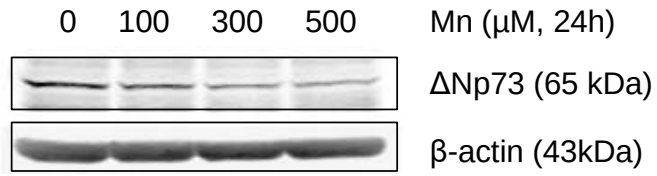


Fig 2: Mn exposure represses ΔNp73 gene and protein expression in N27 dopaminergic cells. N27 dopaminergic cells were exposed to 300 µM of Mn for 24 h in reduced sera. p73 mRNA expression was examined by qRT-PCR (A). N27 cells were exposed to 300 µM of Mn in a time course over 24 h. Expression of ΔNp73 protein was measured by Western blot assays (B). Densitometric analysis was performed (C). Data are expressed as percent of control and represented as mean ± S.E.M. from three independent experiments. Asterisks (n=6-9, ***, $p < 0.001$; *,$p<0.05$) indicate significant differences between treatment and control groups.

A



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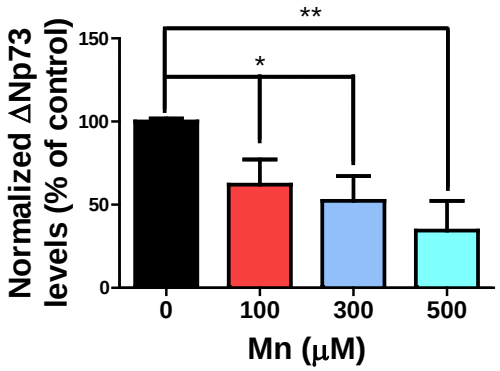

Fig 3: Mn downregulates ΔNp73 in primary striatal neurons. Primary striatal neurons were exposed to 100-500μM of Mn for 24h in reduced sera. Expression of ΔNp73 protein was measured by Western blot assays (A). Densitometric analysis was performed (B). Data are expressed as percent of control and represented as mean ± S.E.M. from three independent experiments. Asterisks (n=6-9, *, $p<0.05$; **, $p<0.01$) indicate significant differences between treatment and control groups.

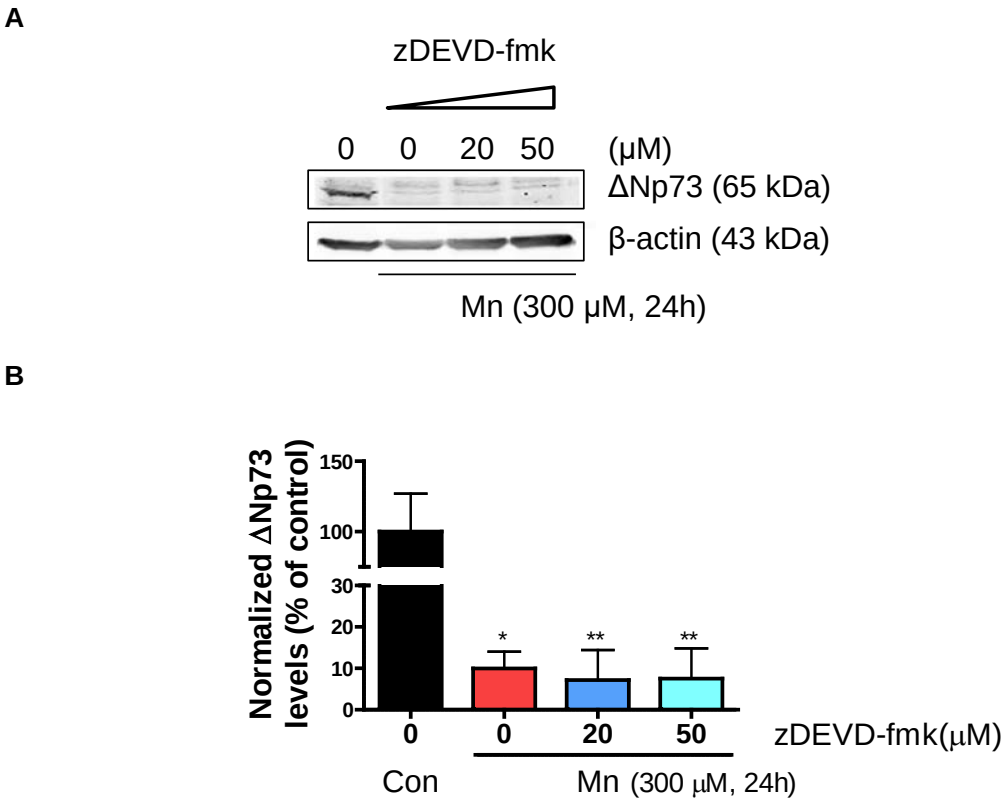

Fig 4: Caspase-3 inhibition does not alter the downregulation of ΔNp73during Mn-induced neurotoxicity. N27 dopaminergic cells were pre-treated with either 20 or 50μM of the caspase-3 inhibitor zDEVD-fmk before exposure to 300 μM of Mn for 24 h in reduced sera. Expression of ΔNp73 protein was measured by Western blot assays (A). Densitometric analysis was performed (B). Data are expressed as percent of control and represented as mean ± S.E.M. from three independent experiments. Asterisks (n=6-9, *, $p < 0.05$; **, $p<0.01$) indicate significant differences between treatment and control groups.

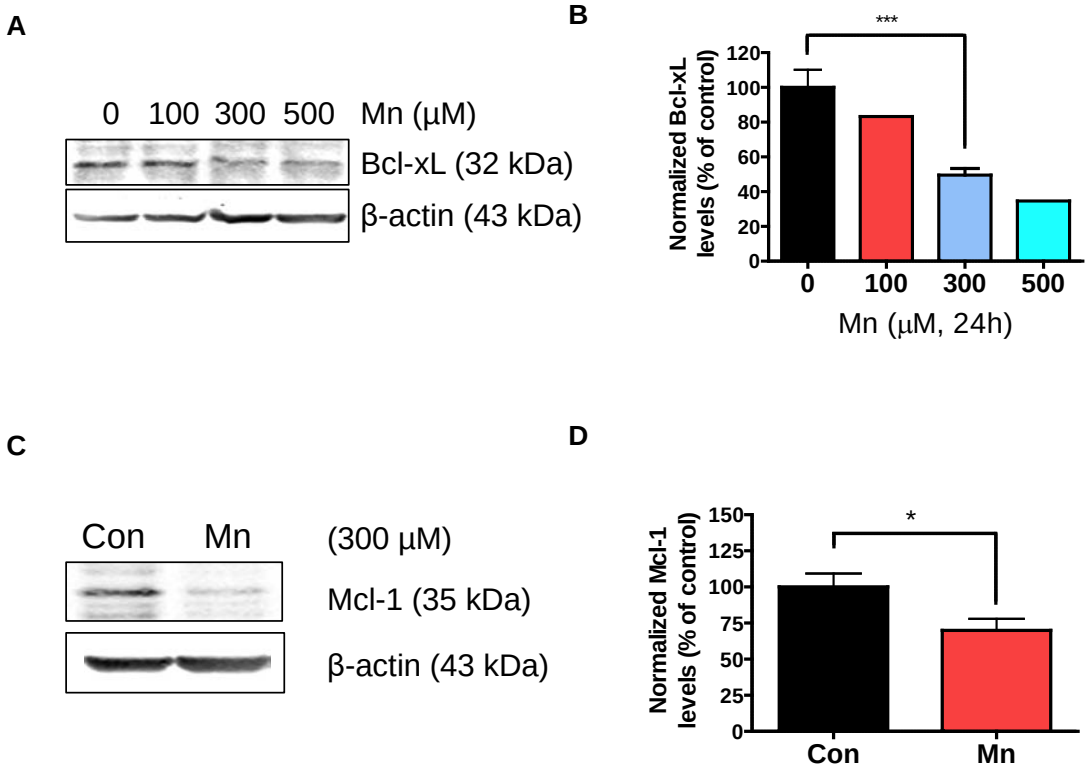

Fig 5: Expression of the downstream effectors of the p73signaling pathway is also downregulated in Mn-induced neurotoxicity. N27 dopaminergic cells were exposed to 100-500μM of Mn for 24h in reduced sera. Expression of Bcl-xL (A) and the longer isoform of MCl-1 (C) was measured by Western blot assays. Densitometric analysis was performed (B and D). Data are expressed as percent of control and represented as mean ± S.E.M. from three independent experiments for Bcl-xL and four independent experiments for Mcl-1, respectively. Asterisks (n=6-9, *, $p < 0.05$; ***,$p<0.001$) indicate significant differences between treatment and control groups.

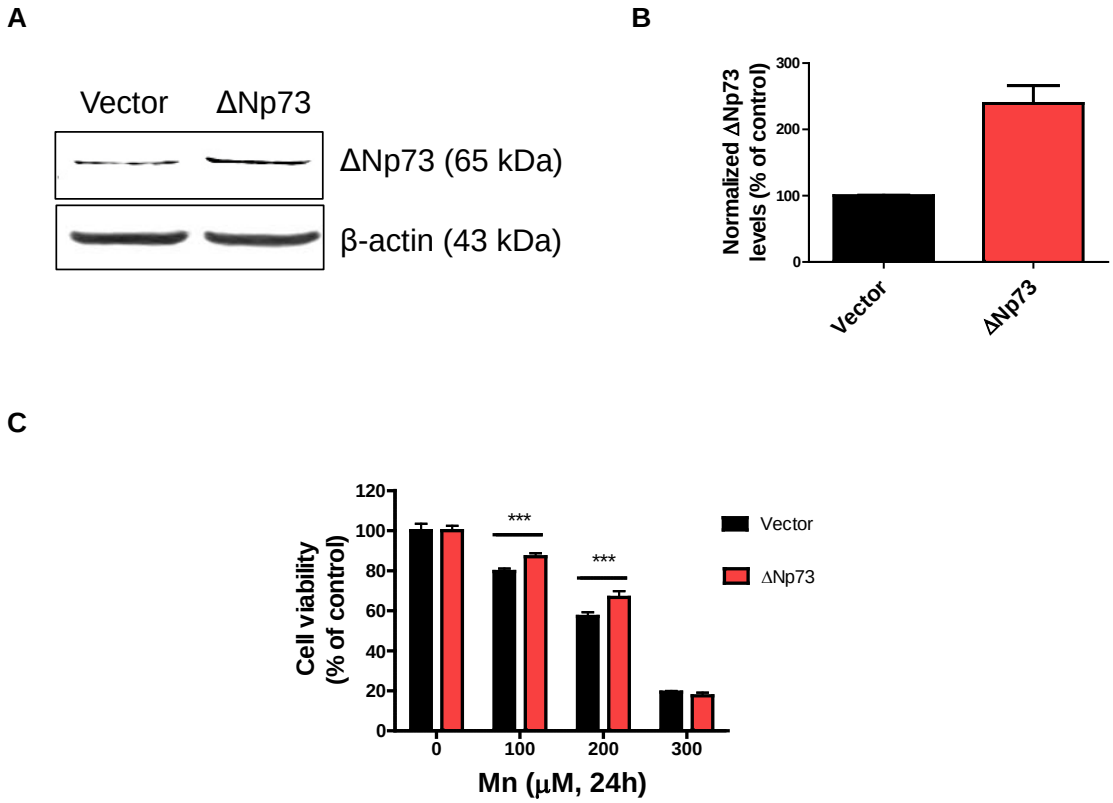

Fig 6: Overexpression of ΔNp73 protects against Mn-induced neurotoxicity. N27 dopaminergic cells were overexpressed with empty vector or ΔNp73. Cells were exposed to 100-300μM of Mn for 24h in reduced sera. Expression of ΔNp73 protein was measured by Western blot assays (A). Densitometric analysis was performed (B). Cell survival was measured by MTS assay (C). Data are expressed as percent of control and represented as mean ± S.E.M. from six independent experiments for MTS assay. Asterisks (n=6-9, ***, $p<0.001$) indicate significant differences between ΔNp73-expressing and empty vector-expressing groups.

CHAPTER III: MANGANESE EXPOSURE DOWNREGULATES STAT5B TO COMPROMISE THE CELLULAR PROTECTIVE MECHANISMS AGAINST MANGANESE NEUROTOXICITY

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Abstract

Manganese (Mn) is an essential element found in most tissues. However, chronic exposure to Mn has been linked to the pathogenesis of manganism, which displays neurological abnormalities somewhat similar to those associated with Parkinson's disease. Yet, the cellular and molecular mechanisms underlying Mn-induced neurotoxicity have not been defined. Recently, we showed that protein kinase C delta (PKCδ) is one of the key mediators of Mn-induced apoptosis in neuronal cells. To further characterize kinase-dependent cell death signaling mechanisms, we examined the effect of Mn on several signaling pathways, including signal transducer and activator of transcription (STAT). Interestingly, a 300 □ M Mn exposure in N27 dopaminergic neuronal cells over 12 h downregulated STAT5B protein levels in a time-dependent manner. However, STAT1 was unaffected during the Mn treatment, indicating an isoform-specific effect of Mn on STAT5B. Quantitative RT-PCR analysis also showed that Mn treatment reduced STAT5B mRNA levels. Exposing primary striatal cultures to Mn also reduced STAT5B. Furthermore, Bcl2, one of the downstream targets of STAT5B, was concomitantly reduced with STAT5B downregulation. Pretreatment of 20 μM Lactacystin failed to protect downregulation of STAT5B indicating STAT5B downregulation was independent of proteasomal degradation pathway. Pre-treatment of N-Acetyl Cystine (NAC) was shown to protect downregulation of STAT5B. In addition, treatment of MPP^+ in N27 cells showed downregulation of STAT5B. These results support the hypothesis that Mn exposure mediates oxidative stress that induces downregulation of STAT5B. Overexpression of STAT5B cells protected N27 cells against Mn-induced neurotoxicity. Furthermore, overexpression of STAT5B protected mitochondria in N27 cells.

Downregulation of STAT5B was recapitulated in substantia nigra of C57 black mice model treated with Mn and MitoPark Parkinson's disease model. Furthermore, we show that Mn exposure suppresses promoter activity of STAT5B in MN9D dopaminergic cells. Finally, human lymphocytes show downregulation of STAT5B during Mn exposure, proposing a potential drug candidate for Mn-induced neurotoxicity and Parkinson's disease patients. Taken together, our results suggest that Mn exposure downregulates STAT5B in striatal neurons and that Mn-induced downregulation of STAT5B may compromise the protective signaling pathway, thereby exacerbating neuronal cell death (NIH grants ES10586, ES19267, NS74443).

1. Introduction

Neurodegenerative diseases have recently become more prevalent possibly due to aging, genetic alterations, and exposure to environmental toxicants such as pesticides, heavy metals and industrial byproducts. Manganese (Mn) has been used widely for more than two centuries in industry for various purposes. The main sources of Mn intake are through the daily diet and occupational exposure to Mn-containing fumes. Airborne Mn particles from various chemicals, including methylcyclopentadienyl manganese tricarbonyl (MMT) and fungicide, are another important source of public exposure (Bowman et al., 2011, Caudle et al., 2012, Michalke and Fernsebner, 2014). Mn is an essential trace element important in bone formation, fat and carbohydrate metabolism, blood sugar regulation, and calcium absorption. Mn serves as a co-factor for a variety of enzymes including superoxide dismutase 2 and glutamine synthetase. However, overexposure to Mn causes a neurodegenerative disorder known as manganism, which is somewhat similar to Parkinson's disease (PD). Many of the pathological symptoms of manganism are also seen in PD patients including gait dysfunction, postural instability, bradykinesia, rigidity, and mask-like face expression. Manganism differs from PD in its lack of response to L-dopa therapy and in its early-phase neuropsychological sequelae. Both manganism and PD exhibit dopaminergic neuronal cell death in the substantia nigra (SN) and striatum (STR).

Protein kinase C□ (PKC□) is a novel redox-sensitive PKC family kinase (Kanthasamy et al., 2003, Kaul et al., 2003). PKC□ □ has been reported as a key mediator of the apoptotic process of various cells. PKC□ is activated under oxidative stress conditions

such as environmental toxicant exposure. For example, when neuronal cells are exposed to excessive Mn, PKC□ □ gets cleaved by Caspase-3, and the catalytic domain of PKC□ gets released from the regulatory domain and phosphorylated. The resulting active form of PKC□ translocates into the nucleus and activates its target proteins to orchestrate a series of processes preceding cellular apoptotic events.

Signal transduction and activated transducer (STAT) is a transcription factor that transduces many extracellular biological signals to the nucleus to regulate specific genes. STAT has seven family members that share high structural homology. Activation of STATs begins with ligands binding to their cell membrane receptors, leading to activation of Janus-kinase (JAK) by phosphorylation. Activated JAK allows the hetero- or homo-dimerization of proximal STATs before activation by phosphorylation. The phosphorylated STATs translocate to the nucleus where the extracellular signaling exerts fine and specific gene regulation (Paukku and Silvennoinen, 2004, Tan and Nevalainen, 2008).

STAT5 was initially recognized as a mouse mammary gland factor before being renamed as STAT5 because of its significant sequence homology with other STAT members. STAT5 has two isoforms, STAT5A and STAT5B, which have a high 90%sequence homology, with their main difference being a sequence in the C-terminal where the transcription activation domain is located. Though STAT5A and STAT5B share their target genes, they have distinctive roles. They have non-redundant target genes, different profiles of interactions with other co-factors, and different cell type-specific expression profiles (Tan and Nevalainen, 2008, Kanai et al., 2014). STAT5B mediates cellular growth and proliferation, while STAT5A mediates the development and differentiation of mammary

glands. STAT5B-/- mice show impaired growth due to the loss of growth hormone (GH) responsiveness while STAT5A-/- mice show impaired mammary gland development due to the loss of prolactin (Prl) signaling (Buitenhuis et al., 2004, Paukku and Silvennoinen, 2004). Knocked-down expression of STAT5B has also been shown to compromise cellular protection against reactive oxygen species (ROS) in peripheral neuronal system(Du et al.).

With much attention focused on Mn-induced neurotoxicity, the question of how dopaminergic neuronal cell death happens at the molecular-cellular levels recently began to be solved. Hilary *et al*. showed that Mn nanoparticles cause dopaminergic neuronal cell death through mitochondrial-dependent apoptotic signaling and autophagy (Afeseh Ngwa et al., 2011). Our group has recently shown that PKC□ is an important key mediator in driving dopaminergic neuronal cell death (Kanthasamy et al., 2003, Kaul et al., 2003, Jin et al., 2011a). However, despite extensive research, we still need to delineate more clearly the mechanisms connecting Mn neurotoxicity and Parkinsonian disorders. In this report, we investigate whether regulation of STAT5B expression may be involved in Mn-induced dopaminergic neuronal cell death.

2. Materials and Methods

2.1 Chemicals

Manganese chloride ($MnCl_2$, 99%), MPP+, and MPTP were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies against Bcl-2 and STAT5B were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-STAT5and STAT1 were purchased from Cell signaling (Danvers, MA). Bradford protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA). Antibody against β-Actin was purchased from Sigma-Aldrich. The anti-

mouse and anti-rabbit secondary antibodies (Alexa Fluor 680 conjugated anti-mouse IgG and IRdye 800 conjugated anti-rabbit IgG) were purchased from Life Technologies (Grand Island, NY) and Rockland (Gilbertsville, PA), respectively. RPMI1640, fetal bovine serum (FBS), L-glutamine, penicillin, and streptomycin were purchased from Life Technologies. MitoTracker Red was purchased from Life Technologies. N-acetyl-L-cysteine was purchased from Sigma-Aldrich (St. Louise, MO). Lactacystin was purchased from Cayman chemical (Ann Arbor, MI). CellTiter® 96 AQueous Assay and Nano-Glo® Luciferase assay system were purchased from Promega (Madison, WI).

2.2 Animal studies

Six- to eight-week-old male C57BL/6J mice were purchased from Charles River (Chicago, IL). MitoPark mice were kindly gifted from Dr. Nils-Goran Larson at the Max Planck Institute for Biology and Ageing in Cologne, Germany. MitoPark mice were maintained, bred and genotyped at Iowa State University (ISU). Animals were housed at RT under a 12-h light cycle with water and food provided *ad libitum*. Mn (30mg/kg) was orally administered daily for 30 days. MPTP (30mg/kg) was administered intraperitoneally for 4 days. Animals were cared for according to protocols approved by the Institutional Animal Care and Use Committee at ISU. After the treatment period, the striatum was dissected for measuring the expression of proteins of interest.

2.3 Cell cultures and treatment

A rat-derived dopaminergic neuronal cell line, N27, was kindly gifted from Dr. Kedar N. Prasad at the University of Colorado Health Sciences Center, Denver, CO (Adams et al., 1996). N27 cells and MN9D cells, a mouse-derived dopaminergic neuronal cell line, were maintained in RPMI 1640 media supplemented with 10% FBS, 2mM L-glutamine, 50 units of penicillin, and 50µg/ml of streptomycin in a humidified incubator with 5% CO_2 at 37°C as described previously(Jin et al., 2011b, Latchoumycandane et al., 2011). After exposing N27 cells to various concentrations of Mn

(100-500 μM) for 24 h, the cells were subjected to quantitative real-time PCR (qRT-PCR) or immunocytochemical analysis. Human lymphocytes from Parkinson's disease patients and population control subjects were purchased from the Corriell Institute for Medical Research (Camden, NJ). Lymphocytes were maintained in RPMI 1640 media supplemented with 15% raw FBS, 2mM L-glutamine, 50units of penicillin, and 50μg/ml of streptomycin in a humidified incubator with 5% CO_2 at 37°C.

2.4 Primary striatal cultures and treatment

The primary striatal neuronal cultures were prepared as described previously from the ganglionic eminences of gestational 14- to 15-day-old mouse embryos(Jin et al., 2014a). Briefly, after removing the embryonic brain, the embryonic cerebral cortex was folded away to expose ganglionic eminences and striatum for dissection, which were then washed in Dulbecco's modified Eagle's media (DMEM) and dissociated with trypsinization. Dissociated cells were seeded into poly-D-lysine (PDL)-coated plates and maintained in neurobasal medium supplemented with B-27 supplements, 500mM L-glutamine, 100 IU/ml penicillin, and 100ug/ml streptomycin in a humidified incubator with 5% CO_2 at 37°C. Four- to six-day-cultured primary striatal neuronal cells were exposed to Mn under various conditions.

2.5 Plasmid constructs

STAT5B expression vector was kindly gifted from Dr. Lobie, Department of Molecular Medicine and Pathology, University of Auckland, New Zealand. For the promoter reporter system, the forward primer (5'- TACTAGTCCTCGAGAGCTTCTTGGCCCAGCCCCCACGCTCTC-3') and reverse primer (5'- TACTAGTCAAGCTTGCCACCACCAGCGCCCCGGGCCCCTC-3') were used to amplify mouse STAT5B promoter regions from mouse genomic DNA. The resulting PCR amplicon was digested with restriction enzyme before ligation to pNL1.1 NLuc vector (Promega).

2.6 Promoter activity assay

MN9D cells were transfected with luciferase vector fused with either STAT5B promoter or empty vector together with lacZ vector using Lipofectamine2000 (Life Sciences) 24 h prior to exposure to Mn. Treated cells were collected and lysed with lysis buffer (Promega) for the measurement of luciferase activity using Nano-glo® Luciferase assay system (Promega). Cell lysates were further analyzed for β-galactosidase activity, which was used for normalization.

2.7 **Transfections**

N27 cells were transfected with 10ug of pcDNA3 vector or pcDNA3-expressing STAT5B through electroporation. The Amaxa Nucleofector instrument was used according to manufacturer's protocol. Transfected cells were seeded in either T-75 flasks or 96-well plates as desired for 24 h before treatment.

2.8 Cell proliferation assays

Cell survival was measured by colorimetric MTS assay (CellTiter® 96 AQueous Assay). Cells were seeded in 96-well plates before exposure to Mn. At the end of treatment, 20% MTS reagent was added to each well. The MTS tetrazolium compound is bio-reduced in living cells to its colored formazan products. After incubating for up to an hour, absorbance at 490nm was measured using a SpectraMax spectrophotometric plate reader (Molecular Devices).

2.9 Quantitative real-time RT-PCR

After treatment, total RNA was extracted from cells or tissues using an Absolutely RNA Mini Extraction Kit (Agilent Technologies, Santa Clara, CA) according to manufacturer's protocol. Total RNA was treated with DNase I during extraction of total RNA and converted to cDNA using the High Capacity cDNA Synthesis Kit (Life Technology, Grand Island, NY).SYBR Green quantitative PCR was performed. Forward primer (5'-GCAATGATTATAGCGGCGAGA-3') and reverse primer (5'-CAAAGGCGTTGTCCCAGAGG-3') were used for detection of STAT5B mRNA. Primers for 18S RNA

(Qiagen) were used as the housekeeping gene (control). The threshold cycle (C_t) was calculated from the instrument software, and fold change of gene expression for pair-wise comparisons was calculated using the$\Delta\Delta C_t$ method.

2.10 Western blotting

Cells were lysed in lysis buffer (1% Triton X-100, 1mM EDTA, 100mM NaCl, 1mM EGTA, 1mM NaF, 20mM $Na_4P_2O_7$, 2mM Na_3VO_4, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 50mM Tris-Cl, pH7.4). Brain homogenates were prepared as described previously (Ghosh et al., 2013).Western blotting was performed as described previously (Jin et al., 2014b). Briefly, the samples containing equal amounts of proteins were fractionated through a 10 to 15% SDS-PAGE gel and transferred onto a nitrocellulose membrane. Primary antibodies against specific proteins were incubated with the membrane. After thoroughly rinsing in PBS supplemented with 0.1% Tween-20, the membrane was incubated with either Alexa Fluor 680 goat anti-mouse, Alexa Fluor 680 donkey anti-goat (Invitrogen, Carlsbad, CA, USA), or IRDye 800 donkey anti-rabbit secondary antibodies (Rockland, Gilbertsville, PA, USA). For loading control, β-Actin was used. Immunoblot imaging was performed with an Odyssey Infrared Imaging system (LI-COR, Lincoln, NE, USA). ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to quantify Western blot bands.

2.11 Mitochondrial dysfunction assay

The mitochondrial dysfunction assay has been previously described (Charli et al., 2015). Briefly, cells were grown on PDL-coated glass slides and exposed to Mn. Cells were washed with PBS and stained with 200nM MitoTracker Red dye diluted in serum-free RPMI media before fixation in 4% paraformaldehyde (PFA) for 30min. Cell images were taken at 60x magnification and captured with a SPOT color digital camera.

2.12 Immunostaining and Microscopy

N27 cells were grown on PDL-coated glass slides and exposed to Mn. The treated N27 cells were fixed and blocked by incubating in 4% PFA for 30min and in blocking buffer (2% bovine serum albumin, 0.5% Triton X-100, 0.05% Tween-20 in PBS), respectively. Cells were incubated overnight at 4°Cwith antibody against STAT5B (1:500, Santa Cruz Biotechnology). After washing, cells were incubated with fluorescently conjugated secondary antibody (Alexa Fluor 555-conjugated anti-mouse antibody, 1:2000) to visualize protein. Hoechst 33342 (10µg/mL) was used for nuclear staining. Images were taken at 60× magnification with a SPOT color digital camera attached to a Nikon TE2000 microscope and processed using ImageJ software.

2.13 Statistical analysis

Data were analyzed using Prism 4.0 software (GraphPad Software, San Diego, CA). Paired Student *t*-tests were performed. Tukey's multiple comparison test was used after ANOVA for comparing differences between multiple groups. Differences were considered statistically significant if p-values < 0.05 were obtained. Data are represented as the mean ± S.E.M. of at least two separate experiments performed at least in triplicate.

3. Results

3.1 Downregulation of STAT5B in dopaminergic neurons after Mn-induced neurotoxicity

Mn neurotoxicity induces activation of protein kinase C (PKC), a novel redox-sensitive PKC family kinase (Kanthasamy et al., 2003, Kaul et al., 2003). PKC has also been reported as a key mediator in the apoptotic process of various cells, making it important to identify its downstream targets during Mn-induced neurotoxicity, for which STAT family members might be one. Unexpectedly, exposing dopaminergic neuronal cells to 300 µM of

Mn downregulated STAT5B expression by over 70% (Fig. 1 A and B). STAT1 expression was not significantly altered compared to control, indicating isoform-specific regulation of STATs during Mn exposure. Expression of STAT5B at the transcriptional level was investigated by performing qRT-PCR. Surprisingly, Mn exposure downregulated STAT5B transcription by more than 80% compared to control (Fig. 1 C). The activated phosphorylated form of STAT5B was also significantly downregulated by more than 50% compared to control. To characterize the subcellular localization of STAT5B during Mn exposure, immunocytochemistry analysis was performed. The amount of STAT5B in the nucleus of Mn-treated N27 cells was significantly lower than in control cells (Fig. 1D).

3.2 Profiles of STAT5B downregulation

We measured the expression of STAT5B at various time points during Mn exposure. N27 cells were exposed to 300 µM of Mn for 3, 6, 9 and 12 h. Downregulation of STAT5B began as early as 3 h after Mn exposure (Fig. 2A). Mn-induced cytotoxicity downregulated STAT5B expression in a time-dependent manner, with peak suppression of STAT5B occurring at 12 h. To preclude the possibility that the Mn-induced STAT5B downregulation was specific to the N27 cell model, the experiment was repeated in another commonly used dopaminergic neuronal cell line, MN9D cells. As with N27 cells, Mn exposure to 300 µM for 12 h resulted in significant downregulation of STAT5B in the MN9D cell culture model (data not shown). The evaluation of expression of STAT5B during Mn exposure was further extended to mouse primary striatal cultures.

Next, we tested the effects of Mn cytotoxicity on mouse primary striatal cultures by exposing them to 0, 100, 200, 300, and 500 µM of Mn for 24 h (Fig. 2C). Again, we observed a dose- and time-dependent downregulation of STAT5B. Exposure to 100 µM Mn significantly downregulated STAT5B expression by more than30%, while 300 µM Mn suppressed expression by roughly 50% (Fig. 2D). Phosphorylated STAT5B can be degraded through the proteasomal degradation pathway, and we used lactacystin to investigate the effects of Mn on the STAT5B proteasomal degradation pathway (Fig. 3C). N27 cells were pre-treated with 20 µM lactacystin 1 h before being exposed to 300 µM Mn. Pretreatment with lactacystin alone did not induce any phenotypic damage to the neuronal cells. Unexpectedly, lactacystin failed to prevent STAT5B downregulation during Mn exposure, indicating the possibility that other systems, including transcriptional regulation, may be involved in suppressing STAT5B expression. Future studies will explore the mechanisms of how Mn exposure induces STAT5B downregulation.

3.3 Effects of downregulation of STAT5B on dopaminergic neurons

Activated STATs induce the expression of their target genes depending on signals, tissue type, and time. We examined the expression of several target genes to unravel the cascade of signaling molecules following Mn exposure in dopaminergic neurons. Bcl-2 was downregulated concomitantly with STAT5B by more than 50% relative to controls 12 h after exposure to 300 µM Mn (Fig. 3A). The dose- and time-dependent downregulation of Bcl-2 began 6 h post-exposure (data not shown). Bcl-2 is known to be anti-apoptotic. Therefore its

downregulation might suppress cellular protective mechanisms against Mn-induced cytotoxicity.

3.4 Suppression of oxidative stress protects STAT5B during Mn exposure

Mn overexposure induces oxidative stress (Erikson et al., 2004). To investigate whether Mn-induced oxidative stress plays a role in STAT5B downregulation, we pre-treated N27 cells with 1mM of the anti-oxidant agent N-acetyl cysteine (NAC) just 1 h before exposure to 300 μM Mn (Fig. 4). The NAC pretreatment induced higher STAT5B expression compared to Mn alone, while NAC alone did not have any effect. To investigate whether excessive oxidative stress can also induce STAT5B downregulation, we treated N27 dopaminergic neuronal cells with 300 μM of the widely known toxicant MPP^+, which causes PD-like symptoms and induces oxidative stress. Treatment with MPP^+ significantly downregulated STAT5B by more than 40% compared to the control group. These results indicate that Mn-induced neurotoxicity may require oxidative stress to induce downregulation of STAT5B expression.

3.5 Overexpression of STAT5B protect neuronal cells in Mn-induced cytotoxicity

We overexpressed the STAT5B protein in dopaminergic neurons before exposing them for 48 h to various amounts of Mn in 2% FBS. Then cell survival was measured using the MTS-based cell proliferation assay, in which metabolically active cells reduce MTS to purple-colored formazan (Fig. 5A). Overexpression of STAT5B significantly protected neuronal cells from exposure to 100 and 200 □ □ Mn, but not 300 □ □ Mn. These results

suggest that sustained STAT5B expression offers cellular protection against Mn-induced neurotoxicity so long as the Mn insult is not too strong. Overexpression of STAT5B also sustained Bcl-2 expression during Mn-exposure, indicating that the protective mechanism may be mediated through Bcl-2-related cell signaling (Fig. 5B).

3.6 Overexpression of STAT5B protects mitochondria in neuronal cells during Mn exposure

One STAT5B function is to regulate energy metabolism and to be involved in mitochondrial regulation (Du et al., 2012). Since the anti-oxidant reagent NAC protected STAT5B from Mn-induced neurotoxicity, we explored the possibility that STAT5B affects mitochondrial biogenesis. N27 dopaminergic neuronal cells overexpressing STAT5B-GFP were stained with MitoTracker red dye after exposure to Mn. Mitochondrial structure was analyzed and quantified with ImageJ software. Mitochondria in control groups showed long thread-like mitochondria distributed randomly in the cytosol. In contrast, mitochondria in GFP-expressing neuronal cells were significantly damaged after Mn exposure, exhibiting a disintegrated and disconnected circular form indicative of mitochondrial fission. Thus, Mn exposure induced mitochondrial damage in dopaminergic neuronal cells overexpressing GFP, however, overexpression of STAT5B-GFP significantly protected mitochondria from Mn-induced neurotoxicity (Fig 6A), allowing the dopaminergic neuronal cells to retain longer thread like mitochondria (Fig 6B).Circularity of mitochondria in STAT5B-GFP-overexpressing cells was lower than in GFP-expressing cells. These results showed that STAT5B plays a role in protecting mitochondria during Mn exposure.

3.7 Downregulation of STAT5B *in vivo*

To investigate whether Mn exposure suppresses STAT5B *in vivo*, C57 black mice were administered 30mg/kg of Mn via oral gavage for 30 days before performing behavioral and biochemical analyses (Fig. 7). The Mn-treated group traveled a shorter distance than did the control group, indicating that Mn exposure affected the SN, which governs body movement (data not shown). The SN was dissected for further biochemical analyses. Expression of STAT5B was measured by Western blot and normalized to β-Actin. Densitometric analysis showed Mn exposure induced more than a 50% downregulation of STAT5B in the SN when compared to the control group.

C57 black mice were treated with 30mg/kg MPTP for 4 days and sacrificed on the 5th day to investigate whether MPTP exposure alters STAT5B expression. Interestingly, MPTP exposure induced more than a 60% downregulation of STAT5B (Fig 8). As STAT5B downregulation was surprisingly responsive to toxicants such as Mn and MPTP, we tested STAT5B expression in yet another PD model known as MitoPark, a progressive genetic mouse model of PD. We compared the STAT5B expression levels of 25-wk-old MitoPark mice to C57 black mice serving as age-matched controls. Densitometric analysis of the Western blot of striatal tissue lysates showed more than a 50% reduction of STAT5B expression in MitoPark mice (Fig. 9). We further analyzed STAT5B expression in different age groups. MitoPark mice begin to exhibit reduced STAT5B in the SN at 12 wk and STAT5B expression continues to progressively degenerate as they age (data not shown).These results show that STAT5B expression was responsive to toxicants, which induced its downregulation *in vivo*.

3. 8 Promoter 1 activity of mouse STAT5B was repressed during Mn exposure

The finding that the mRNA level of STAT5B was downregulated during Mn exposure prompted us to investigate its promoter activity during Mn exposure in mice. We cloned the STAT5B promoter 1 region from 1000 bp upstream to 500 bp downstream and attached a Luciferase reporter gene. We transfected the resulting mini STAT5B promoter reporter gene into MN9D cells, which is another well-known mouse dopaminergic neuronal cell line. After a 9h exposure to 300μM Mn, promoter activity was suppressed by more than 50%, which indicates that Mn-induced transcriptional regulation of STAT5B plays an important role in downregulating STAT5B expression. The response of promoter activity to Mn exposure remains to be characterized.

3.9 STAT5B as potential biomarker for Mn exposure

The high sensitivity of dopaminergic neurons to Mn prompted us to explore whether STAT5B could serve as a biomarker for PD-like symptoms including manganism. For this study, we compared human lymphocytes from both PD patients and a population control group. PD patients were 59-60 years old. Human lymphocytes were exposed for 24 h to various concentration of Mn ranging from 100μM to 500μM, and then we measured cell survival using the trypan blue assay in a Vi-CELL cell viability analyzer. The lower concentration of 100 μM Mn induced 50% cell death in reduced serum-complemented media (data not shown). STAT5B was downregulated in Mn-treated lymphocytes for both the population control and PD patient groups. These results show that STAT5B expression is

very sensitive to Mn exposure in lymphocytes and may serve as a biomarker for Mn-induced toxicity.

4. Discussion

Manganese's versatile redox states ranging from Mn^{2+} to Mn^{7+} serve important roles in many biochemical reactions in most living organisms including mammals. However, overexposure to Mn promotes the development of neurodegenerative diseases similar to idiopathic PD. Yet it remains unclear how Mn exposure causes neuronal cell death. In the present study, we demonstrate that Mn exposure downregulates the native and phosphorylated forms of STAT5B at both the protein and mRNA levels (Fig. 1), while STAT1 expression remains stable. Thus, Mn exposure appears to selectively downregulate STAT5B, suggesting that STAT5B plays important roles in Mn-induced neurotoxicity. STAT5B becomes functional or activated when it is phosphorylated at Y699, as Y699-phosphorylated STAT5B (pSTAT5B) is transported to the nucleus where it activates its downstream effector molecules. We determined that Mn exposure indeed reduced pSTAT5B in N27 dopaminergic neuronal cells. Immunocytochemistry (ICC) of the subcellular localization of STAT5B revealed that nuclear STAT5B was also downregulated in N27 dopaminergic neuronal cells during Mn exposure. In addition to expression levels of the STAT5B protein, mRNA levels of STAT5B were also downregulated during Mn exposure, as quantitative RT-PCR results showed.

STATs play important roles in transducing and activating extracellular signals (Paukku and Silvennoinen, 2004). Receptors activated by extracellular signals such as

hormones and cytokines in turn induce phosphorylation of STAT molecules which act as transcription activators. In most cases, cells regulate the action of STAT5B by modulating its phosphorylation status through many mechanisms including phosphatase and PIAS. Interestingly, our results show that Mn exposure induces the downregulation of both the native and phosphorylated forms of STAT5B, which is a novel way of modulating STAT5B functions. STAT5B downregulation occurred in a dose- and time-dependent manner, with a significant drop in protein levels beginning as early as 6h after Mn exposure. Similarly, ICC results also showed nuclear STAT5B to be downregulated within 6h. Wang et al. (Wang et al., 2000) showed that the protease inhibitor MG132 and the phosphatase inhibitor sodium orthovanadate (SO) each protected IL-3-dependent myeloid cells against the downregulation of pSTAT5a, though SO did so to a lesser degree. To confirm that the STAT5B downregulation was not specific to one neuronal cell line, we also examined the effects of Mn exposure on other neuronal cell cultures. The mouse dopaminergic neuronal MN9D cell line exhibited similar STAT5B downregulation during Mn exposure (data not shown). Furthermore, primary mesencephalic striatal cells were cultured from E14 embryonic pups and exposed to various concentration of Mn for 24h. Similar to N27 cells, mouse primary striatal cell cultures showed STAT5B downregulation in a dose-dependent manner (Fig. 2 C). STAT5A has been shown to be regulated by proteasomal proteolysis, although the investigators acknowledged that mishandling of samples may have contributed to the degradation process(Wang et al., 2000, Ramos et al., 2007). To determine whether STAT5B might be degraded via proteasome-dependent proteolysis, we used lactacystin to inhibit proteasomal protein degradation. Pre-treatment with lactacystin failed to protect against

STAT5B downregulation during Mn exposure, indicating that another system mediates the downregulation of STAT5B.

It is possible that microRNA plays a role in regulating STAT5B expression, whose decrease in myometrial tissues during pregnancy has been shown to be mediated by miR-200a (Williams et al., 2012). However, whether similar miRNA-mediated suppression of STAT5B occurs in other cell types like neurons still needs to be explored. Mouse STAT5B has two alternative promoters. Promoter 1 shares high homology with both the human and rat promoter, whereas promoter 2 is exclusive to the mouse. The human STAT5B promoter has SP1 binding sites and methylation-associated CpG binding sites (Crispi et al., 2004). It is possible that Mn exposure disrupts transcription factor activity to reduce transcription of STAT5B. When we examined downstream targets of STAT5B, Bcl-2 was concomitantly downregulated with STAT5B. Bcl-2 has been known to play an anti-apoptotic role and to initiate protective mechanisms. In this case, Mn exacerbates its cytotoxicity by turning down the anti-apoptotic STAT5B pathway. Characterization of its systemic mechanisms may reveal potential drug targets to alleviate or prevent Mn-induced neurodegeneration.

One way Mn exerts its cytotoxicity is to generate reactive oxygen species (ROS). Hence, we examined whether ROS plays a role in STAT5B downregulation by pre-treating N27 cells with 1 mM NAC, an anti-oxidative stress agent, 1 h before a 300 μM Mn treatment lasting 9h. We chose a 9 h post-Mn time point to allow for NAC's protective effect before major Mn-induced neurotoxicity was manifested. Surprisingly, NAC significantly protected STAT5B expression against Mn-induced neurotoxicity as the NAC treated groups showed roughly double the amount of STAT5B expression when compared to Mn alone.

Furthermore, we challenged N27 dopaminergic neuronal cells with MPP^+, which is a widely known toxicant that produces ROS, thereby inducing oxidative stress leading to neurodegeneration. Treatment with 300 μM MPP^+ for 12 h downregulated STAT5B by more than 40%, indicating that oxidative stress plays an important role in regulating STAT5B expression.

To investigate specific roles of STAT5B in Mn-induced neurotoxicity, STAT5B was overexpressed in N27 dopaminergic neurons. Our cell viability assay showed that STAT5B contributes significantly to protecting neurons from Mn-induced neurotoxicity. Bcl-2, a target substrate of STAT5B, was also protected from Mn-induced suppression, showing higher expression levels under STAT5B overexpression, supporting our hypothesis that STAT5B is protective during Mn exposure. Bcl-2 suppresses the release of cytochrome C, which plays a pivotal role in inducing mitochondria-mediated apoptosis. Thus, it is conceivable that overexpressing STAT5B elicits protective effects by retaining higher levels of Bcl-2. Furthermore, mitochondrial staining by MitoTracker revealed that STAT5B overexpression supported relatively healthier mitochondria compared to Mn-treated group (Fig. 6 A). Mitochondria are long and thread-like under normal conditions. However, Mn exposure resulted in mitochondria becoming disconnected and circular in shape. In contrast, STAT5B overexpression helped maintain longer mitochondria with a lower degree of circularity (Fig. 6B and C). Furthermore, STAT5B overexpression induced a higher mitochondrial DNA copy number of mitochondrial D loop regions (Fig 6D). Mitochondrial DNA replication is initiated at D loop regions, which are non-coding regions interact with cis- and trans- factors such as nuclear respiratory factors (NRF). Mitochondria react to

cellular stress such as oxidative stress by increasing mitochondrial DNA copy number and mass. For instance, rotenone treatment can induce a two-fold increase of mitochondrial DNA, while H_2O_2 treatment can upregulate nuclear and mitochondrial genes. Thus, overexpression of STAT5B revives mitochondrial protection mechanisms.

Downregulation of STAT5B was further characterized *in vivo*. C57 black mice exposed orally to 30 mg/kg Mn for 30days showed significant downregulation of STAT5B in the SN (Fig. 7), where Mn accumulates more than in other brain regions. The degeneration of dopaminergic neurons is a major cause of motor behavioral dysregulation in both PD patients and Mn-exposed patients. In addition, 30 mg/kg MPTP also downregulated STAT5B in the SN. These *in vivo* results further support our hypothesis that Mn downregulates STAT5B protein and its functions to exert its cytotoxicity. In MitoPark mice, which have TFAM knocked out under the DAT promoter, striatal STAT5B expression was exhibited in 25-week-old mice. Unlike other toxin-related PD models, MitoPark mice show gradual, progressive dopaminergic neuronal degeneration. Interestingly, MitoPark mice also showed progressive downregulation of STAT5B in the SN (data not shown). These results taken together demonstrate that Mn downregulates native and phosphorylated forms of STAT5B.

We demonstrated that oxidative stress induces the downregulation of STAT5B expression in a Mn dose- and time-dependent manner. The *in vitro* findings were reaffirmed in several animal models. Then we asked if STAT5B can serve as a potential candidate for biomarkers for neurotoxicant-induced oxidative stress. The serum level of Mn does not reliably reflect actual Mn exposure since both the range of serum Mn levels varies and other tissues tend to absorb Mn for long durations (Zheng et al., 2011). However, changes in

STAT5B expression reflect Mn cytotoxicity. In addition, quantitative measurement of STAT5B at the mRNA level negates having to rely on complex procedures and tools such as size exclusion chromatography and inductively coupled plasma mass spectrometry.

In conclusion, this report shows novel mechanisms of downregulation of signaling molecules that contribute to dopaminergic neuronal loss. Our work also provides a novel therapeutic target for Parkinsonian patients as many drugs for the STAT5 molecule have been developed.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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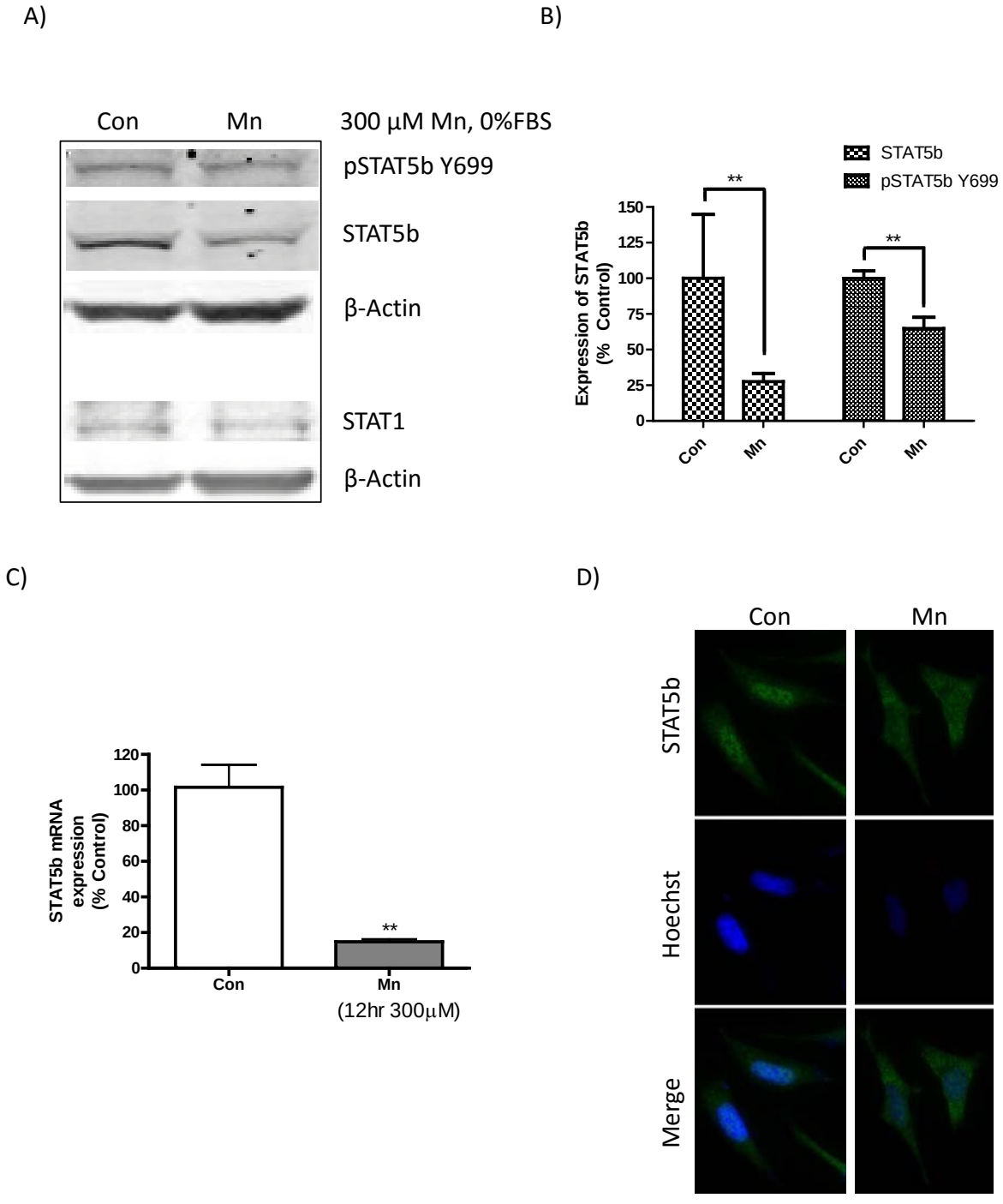

Fig. 1. Manganese (Mn) induces STAT5B downregulation in dopaminergic neuronal cells. N27 dopaminergic neuronal cells were exposed to Mn. Expression of STATs was

measured by Western blot (A) and their densitometric analyses (B). Quantitative expression of STAT5B mRNA post-Mn exposure (C). Intracellular localization of STAT5B post-Mn exposure. Asterisks, **$p<0.01$ for control versus Mn-treated group. The results are represented as the mean ±S.E.M. of three individual replicates of experiments.

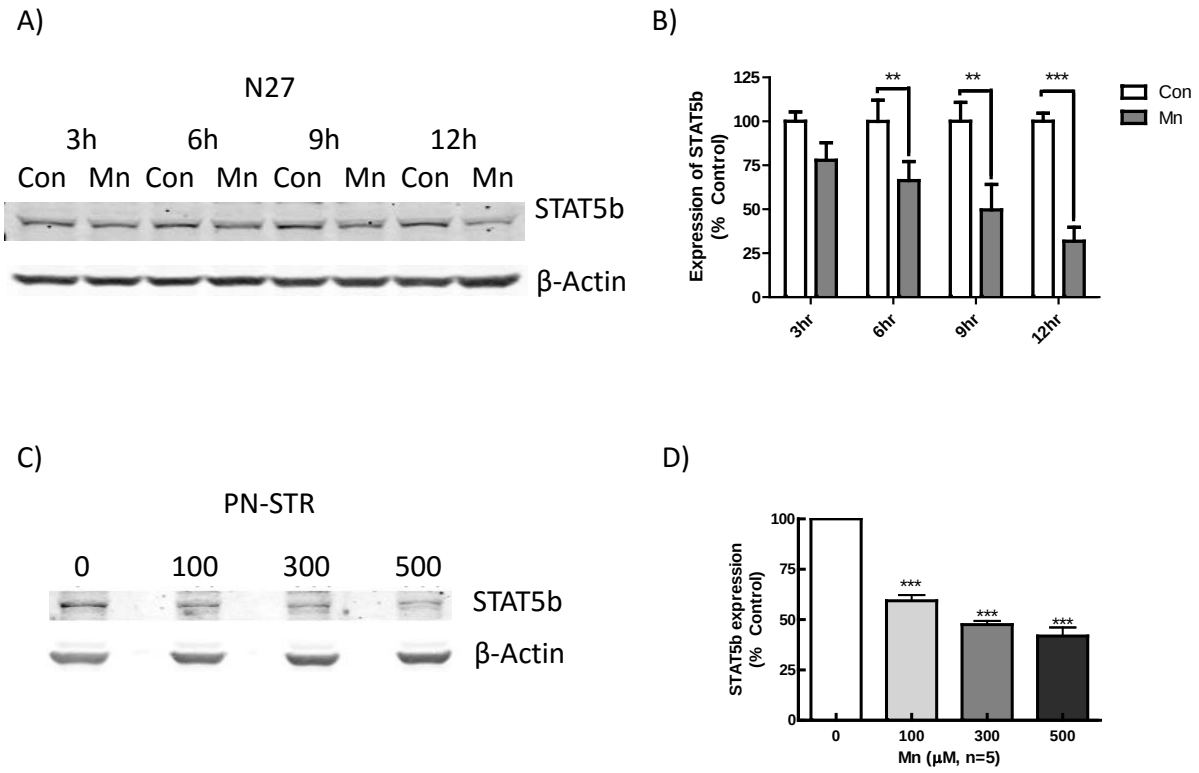

Fig. 2. Time- and dose-dependent downregulation of STAT5B. N27 dopaminergic neuronal cells were exposed to 300μM Mn at various time points (A). Mouse embryonic primary striatal cells were exposed to various concentration of Mn (C). Densitometric analyses (B and D). Asterisks, **p<0.01, and ***p<0.001 for control versus Mn-treated group. The results represent the mean ±S.E.M. of three individual replicates of experiments.

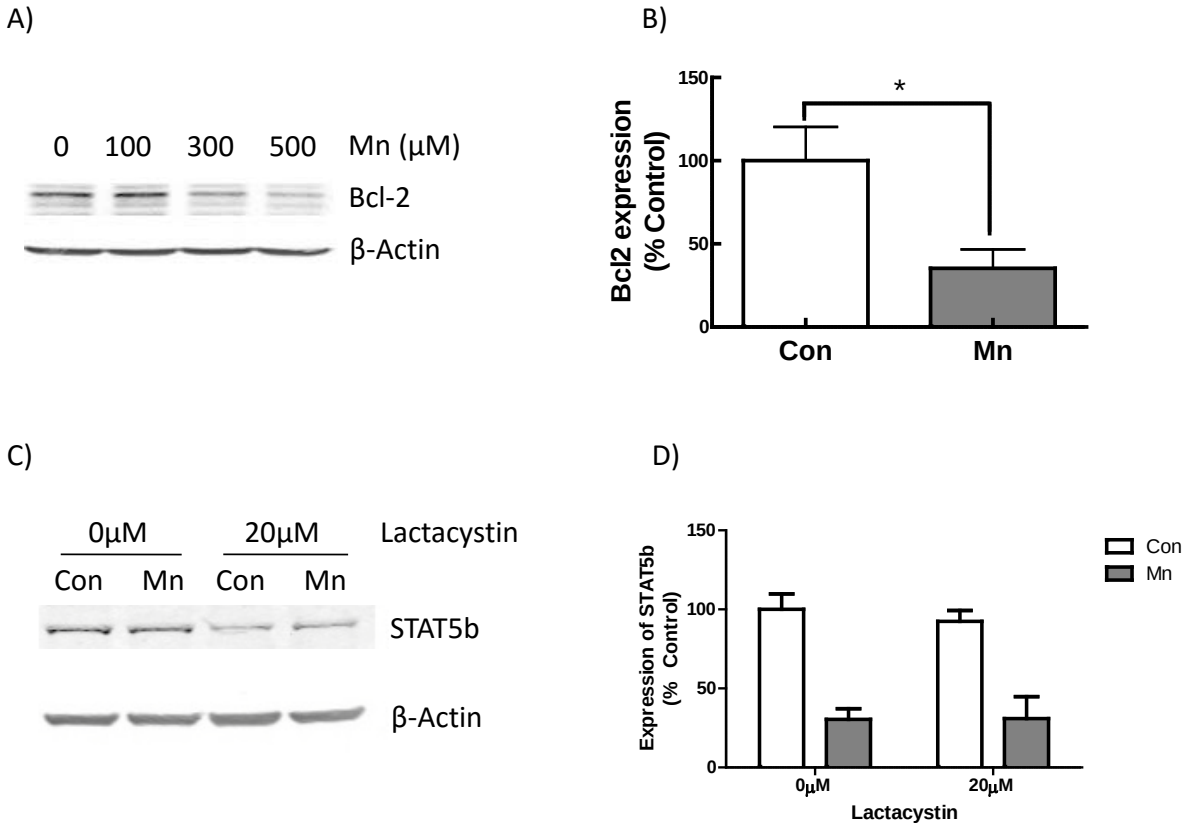

Fig. 3. Manganese induces downregulation of STAT5B pathway. N27 cells were exposed to various concentrations of Mn. Expression of the STAT5B downstream target Bcl-2(A) and densitometric analysis ofBcl-2 expression after 300μM Mn (B). To assess the role of proteasome in downregulation of STAT5B, cells were pre-treated with lactacyst in prior to 300μM Mn exposure (C). Densitometric analysis after lactacystin and Mn (D). Asterisks, *$p<0.05$ for control versus Mn-treated group. The results represent the mean ±S.E.M. of three individual replicates of experiments.

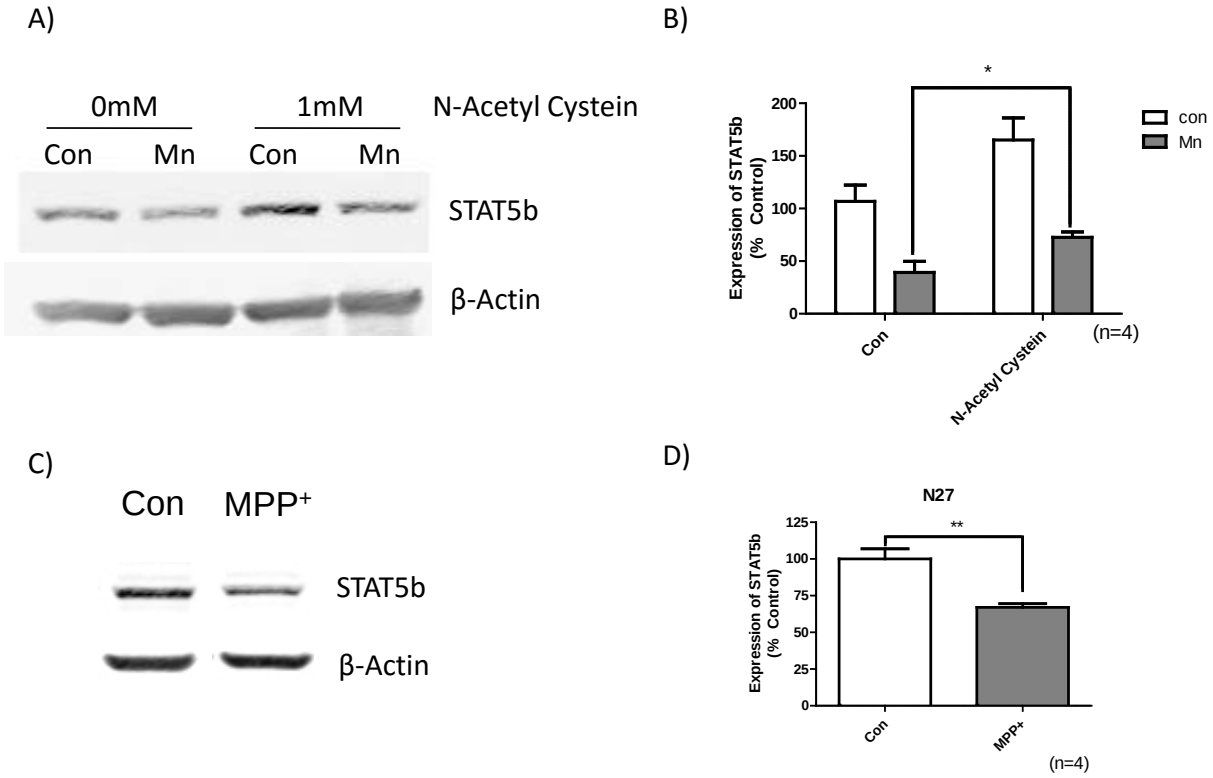

Fig. 4. Oxidative stress induces STAT5B downregulation during Mn exposure. N27 dopaminergic neuronal cells were pre-treated with the anti-oxidative stress agentN-Acetyl Cysteine (NAC) prior to 300µM Mn. STAT5B expression was examined in Western blot (A) and its densitometric analysis (B). The oxidative stress inducer MPP+ was administered to N27 cells and STAT5B expression was measured by Western blot (C) and its densitometric analysis (D). Asterisks, *$p<0.05$ and **$p<0.01$ for control versus Mn-treated group. The results represent the mean ±S.E.M. of three individual replicates of experiments.

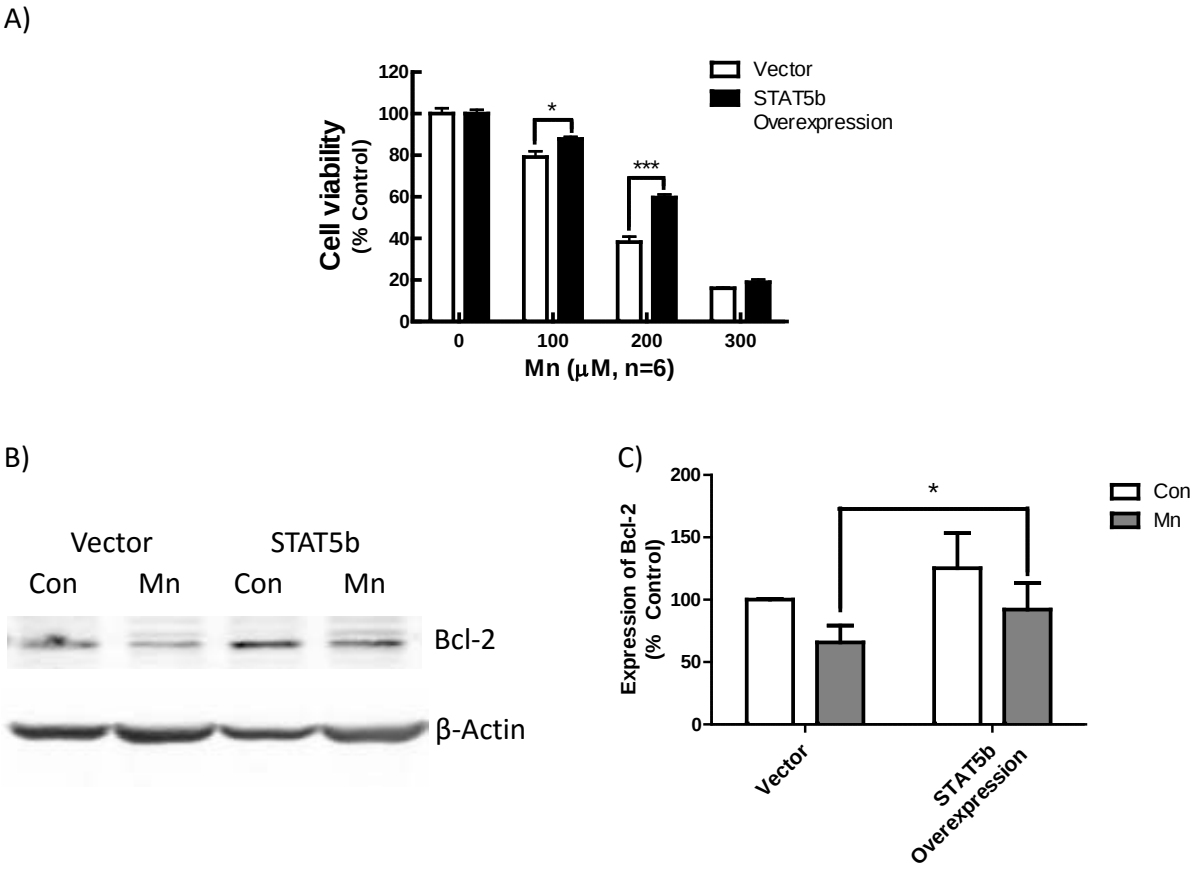

Fig. 5. Overexpression of STAT5B protects N27 dopaminergic neuronal cells from Mn exposure. Cell viability was measured in MTS assay (A). Bcl-2 expression was measured in STAT5B-overexpressed N27 cells (B) and its densitometric analysis (C). Asterisks, *p<0.05 and ***p<0.001 for control versus Mn-treated group. The results represent the mean ±S.E.M. of three individual replicates of experiments.

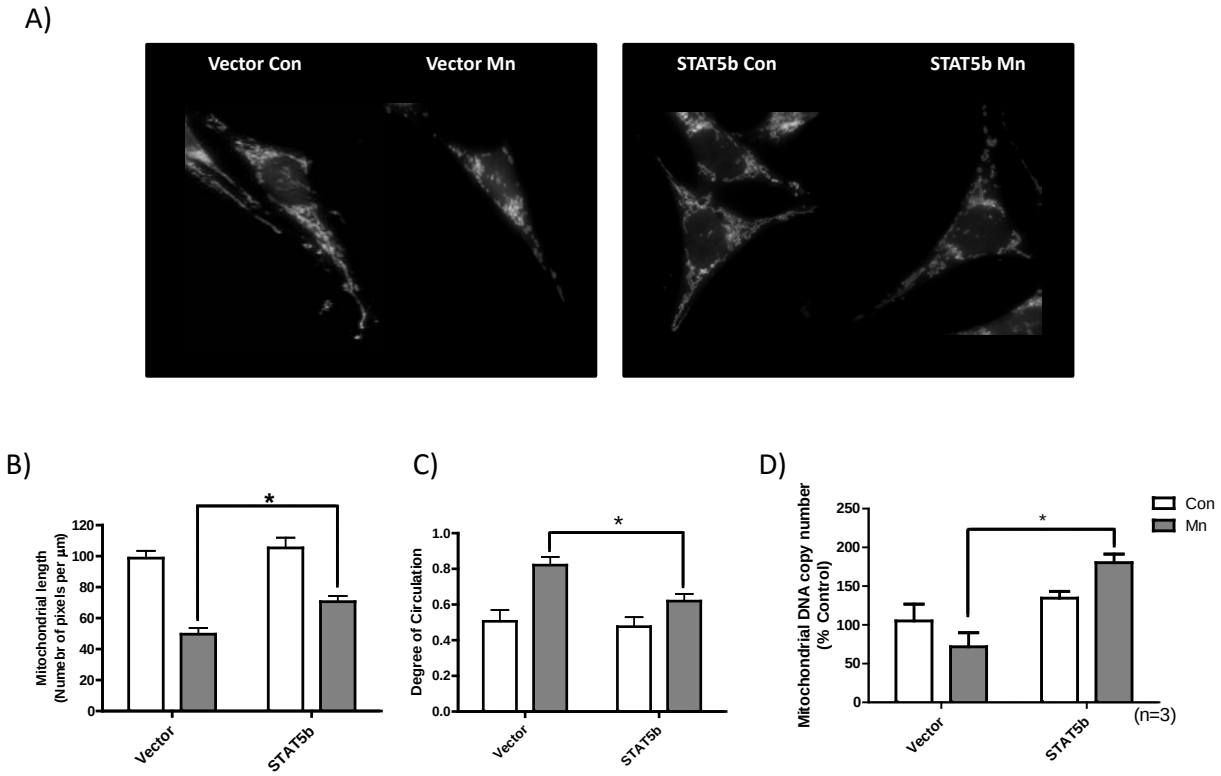

Fig. 6. Overexpression of STAT5B protects mitochondria from Mn-induced toxicity. Mitochondria were visualized in N27 dopaminergic neuronal cells overexpressing STAT5B during a 300 µM Mn exposure (A) for quantifyingmitochondrial length (B), degree of circularity (C) and mitochondrial DNA replication (D). Asterisks, *$p<0.05$ for control versus Mn-treated group. The results represent the mean ±S.E.M. of three individual replicates of experiments.

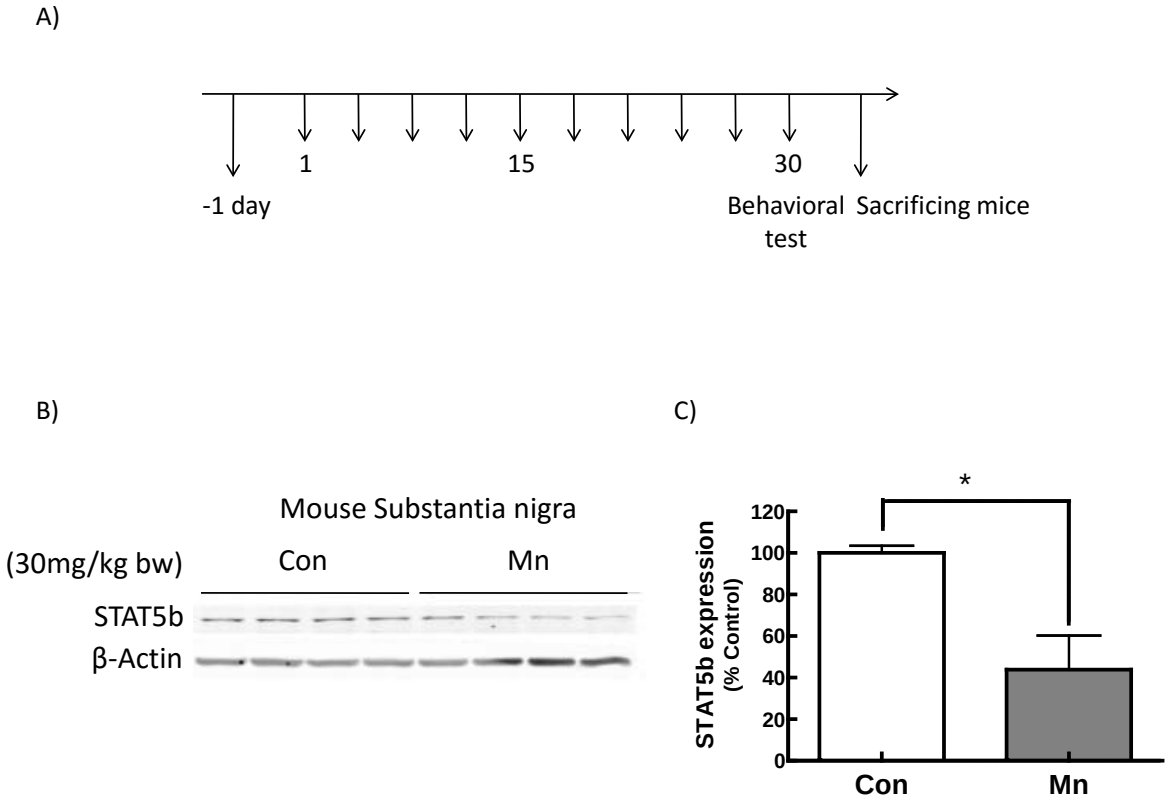

Fig. 7. Expression of STAT5B was suppressed in an *in vivo* model of Mn-induced neurotoxicity. Scheme of Mn treatment to C57 black mice (A).Expression of STAT5B in the substantia nigra in Mn-treated group (B) and its densitometric analysis (C). Asterisks, *p<0.05 for control versus Mn-treated group. The results represent the mean ±S.E.M. of three individual replicates of experiments.

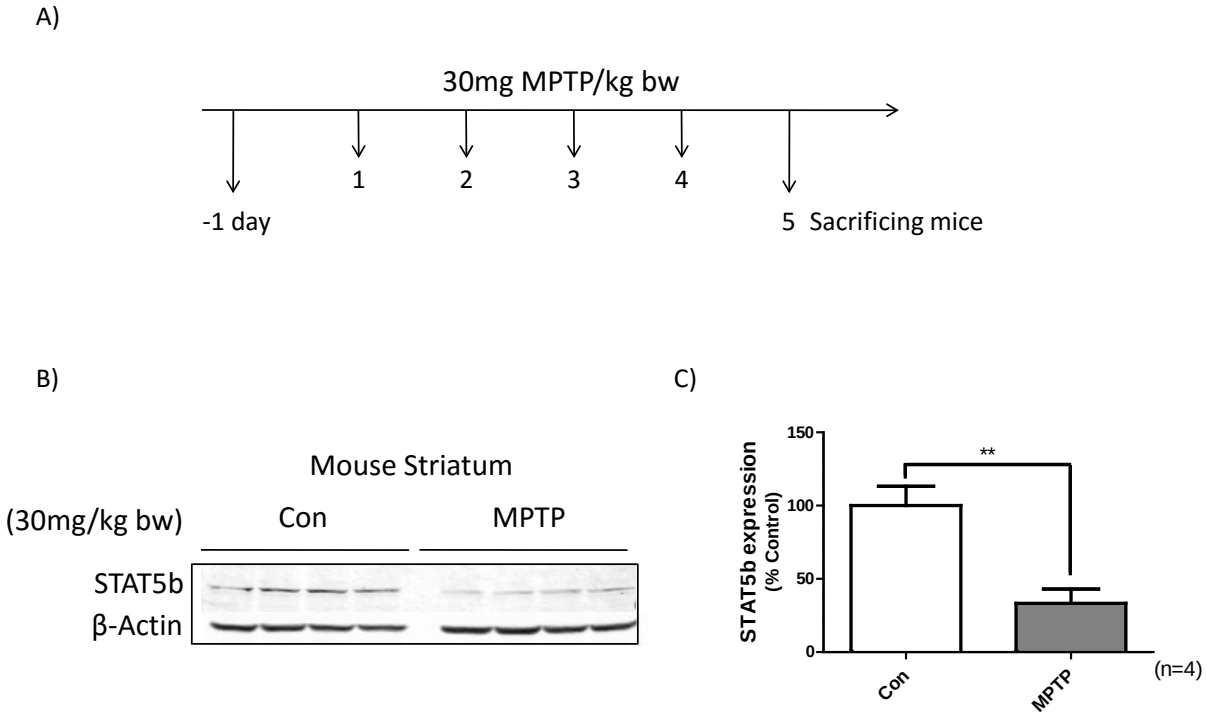

Fig. 8. Expression of STAT5B was suppressed in an *in vivo* model of MPTP-induced neurotoxicity. Scheme of MPTP treatment of C57 black mice (A). Expression of STAT5B in substantia nigra (B) and its densitometric analysis (C). Asterisks, **p<0.01 for control versus MPTP-treated group. The results represent the mean ±S.E.M. of three individual replicates of experiments.

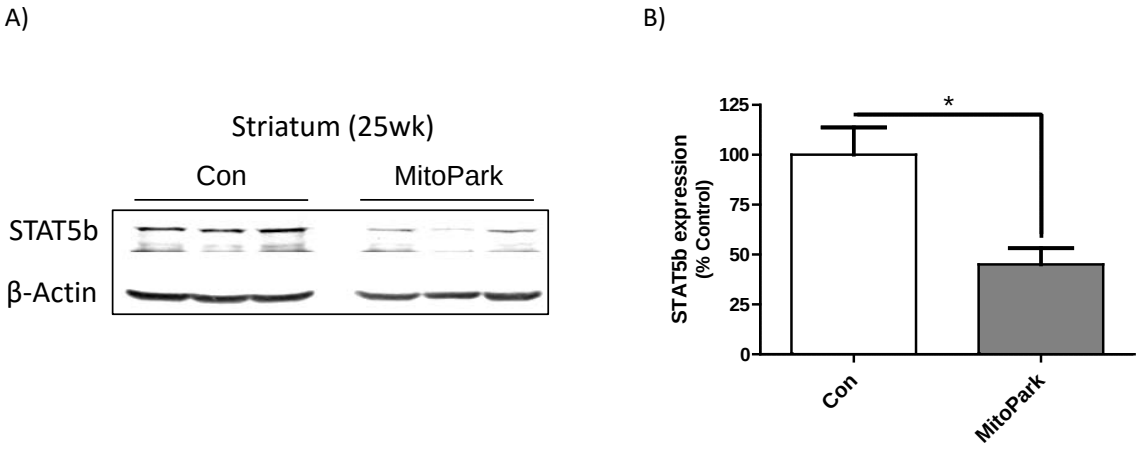

Fig. 9. Expression of STAT5B was suppressed in the MitopPark mouse model of PD. Expression of STAT5B in substantia nigra of 25-wk-old MitoPark mice (A) and its densitometric analysis (B). Asterisks, *$p<0.05$ for control versus MitoPark mice. The results represent the mean ±S.E.M. of three individual replicates of experiments.

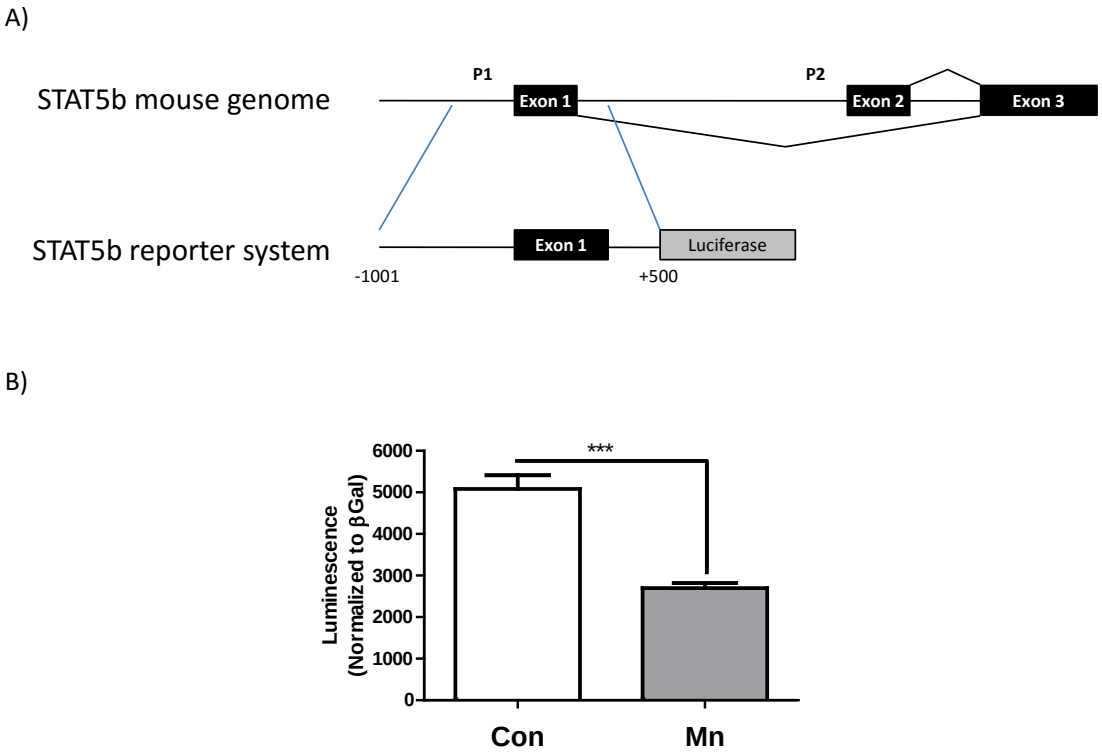

Fig. 10. Manganese induces suppression of STAT5B promoter activity. Scheme of promoter activity reporter system (A). STAT5B promoter from 1000 upstream to 500 downstream was cloned into luciferase reporter system. STAT5B promoter during Mn exposure was measured and quantified (B). Asterisks, ***p<0.001 for control versus Mn-treated group. The results represent the mean ±S.E.M. of three individual replicates of experiments.

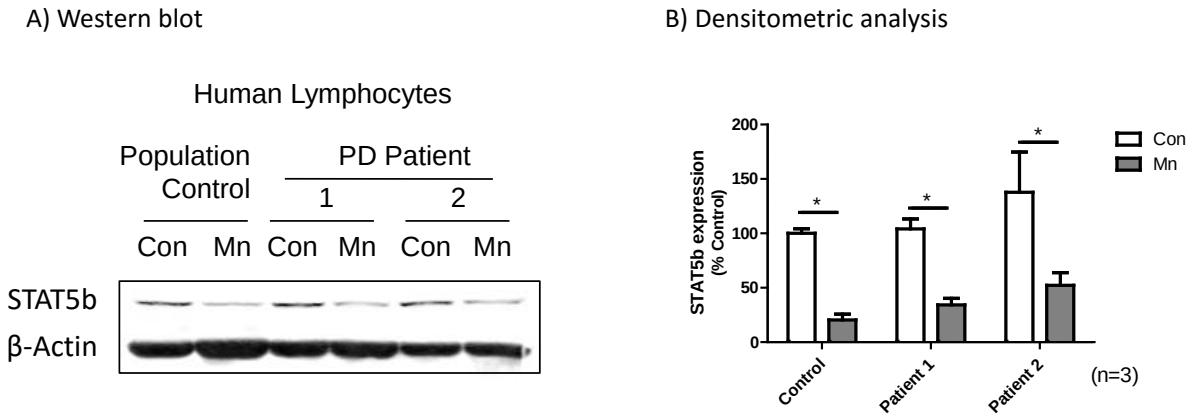

Fig. 11. Manganese exposure downregulates STAT5Bexpression in human lymphocytes. Expression of STAT5B was measured in Western blot for Mn-induced cytotoxicity in human lymphocytes (A) and in its densitometric analysis (B). Asterisks, *$p<0.05$ for control versus Mn-treated group. The results represent the mean ±S.E.M. of three individual replicates of experiments.

CHAPTER IV: MANGANESE EXPOSURE TRANSCRIPTIONALLY SUPPRESSES ANTI-APOPTOTIC STAT5B SIGNALING THROUGH KLF TRANSCRIPTION FACTORS

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Abstract

Chronic exposure to an elevated level of manganese (Mn) has been linked environmentally to Parkinson's disease-like neurological abnormalities. We previously found that Mn exposure selectively downregulated the neuroprotective signal transducer and transcription activator 5b (STAT5B). To characterize the molecular mechanisms underlying STAT5B downregulation in Mn-induced neurotoxicity, the STAT5B promoter was used to examine the effects of 300 μM Mn exposure to MN9D dopaminergic neurons. Promoter analyses revealed that a proximal promoter region near exon 1 contains the regulatory element responsible for the response to Mn exposure to control STAT5B transcription. Detailed site-directed mutational analyses of the putative transcription factor binding sites showed that Sp1 like transcription factor binding sites in the promoter region containing exon 1 may be required for the suppression of STAT5B in Mn-induced neurotoxicity. Two KLF binding sites exhibited inducible transcription repression of STAT5B with Mn exposure, whereas one Sp1 binding site exhibited transcription activation by sensing Mn exposure and reducing its activity. Taken together, these data suggest Mn exposure alters the activities of transcription factors to suppress the transcription of anti-apoptotic STAT5B via an Sp1-like transcription factor-dependent mechanism in dopaminergic neurons, which may significantly contribute to Mn neurotoxicity. (NIH grants ES10586, ES19267, and NS74443).

1. Introduction

Advancement of overall biomedical technology and sciences in the contemporary era increased not only the quality of life but also increased life expectancy. Consequently, an increased elderly population leads more prevalence of neurodegenerative diseases in societies (Duncan, 2011).

Parkinson's disease (PD) is the second most prevalent neurodegenerative disease after Alzheimer's disease. Degeneration of dopaminergic neurons in the substantia nigra region induces motor-behavioral abnormalities as well as other characteristics such as sleeplessness and psychiatric problems. Pathological features include bradykinesia, trembling, rigidity, and postural instability. The exact pathological cause of PD still remains elusive (Gupta et al., 2008, Ben-David and Tu, 2015, Fernandez, 2015).

Manganese (Mn) is a transition metal which has been used in industry for many years. Broad usage of Mn has lead to an increase in human exposure both in industrial settings and everyday lives, from the metal alloy industry to anti-fungal drugs and gasoline additives to medical equipment (Portier, 2012, Farina et al., 2013, Martinez-Finley et al., 2013). Long term exposure to low levels of Mn induces a neurodegenerative disease somewhat similar to PD and shares common pathological features, such as loss of dopaminergic neurons in midbrain leading to abnormal motor behavioral deficit (Caudle et al., 2012, Racette et al., 2012, Farina et al., 2013). Manganese-induced neurodegeneration differs from PD in several ways including inefficiency of L-DOPA treatment (Guilarte, 2011). Nonetheless, Mn exposure plays a very important role in the development of PD, as exposure to Mn expedites PD disease progression (Racette et al., 2001, Santamaria et al., 2007). Despite the emphasis

on finding key molecules that induce degeneration of dopaminergic neurons, few key molecules have been identified to delineate the cellular and molecular mechanisms underlying how Mn exposure induces neurodegenerative diseases.

Signal transducers and activators of transcription (STATs) relays extracellular signals and amplify signaling in downstream cascades (Paukku and Silvennoinen, 2004, Tan and Nevalainen, 2008). STATs are comprised of 6 family members that share high structural homology. Each member of the STAT family has similar or distinct functions. A variety of ligands such as growth hormones, interleukins and cytokines can bind and activate Janus kinase (JAK)s, which in turn recruits and activates STAT proteins by phosphorylation. The homo- or hetero-dimers of phosphorylated STATs are translocated to the nucleus and induces transcription of its target genes. Among STATs, STAT5 plays important roles in growth and differentiation of several tissues (Buitenhuis et al., 2004, Paukku and Silvennoinen, 2004, Abroun et al., 2015). There are two highly homologous STAT5 proteins, STAT5a and STAT5B. STAT5B plays important roles in growth and differentiation of lymphocytes and development, whereas STAT5a plays a role in development and differentiation of mammary gland and female specific gene expressions (Paukku and Silvennoinen, 2004). In spite of distinct roles between STAT5a and STAT5B, they share their gene targets and can somewhat complement each other when either STAT5a or STAT5B is knocked out. In previous studies, our laboratory found that Mn exposure induces downregulation of STAT5B in neuronal cell lines and animal models (unpublished data). STAT5B demonstrated a neuroprotective role against Mn-induced neurotoxicity, indicating downregulation of STAT5B sensitized neuronal cells to Mn-induced cytotoxicity. Overexpression studies showed that STAT5B has

protective effects against to Mn-induced neurotoxicity. Interestingly, we observed the suppression of mRNA expression of STAT5B during Mn exposure. Furthermore, we showed that Mn exposure suppressed transcription of STAT5B. However, the exact mechanisms of how Mn exposure induces downregulation of STAT5B have not been studied. In this study, we report that Mn exposure induces alteration of activities of multiple transcription factors to control of expression of STAT5B.

2. Materials and Methods

2.1 Chemicals

Manganese chloride ($MnCl_2$, 99%) was purchased from Sigma-Aldrich (St. Louis, MO). Antibodies against STAT5B and β-Actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Bradford protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA). The anti-mouse and anti-rabbit secondary antibodies (Alexa Fluor 680 conjugated anti-mouse IgG and IRdye 800 conjugated anti-rabbit IgG) were purchased from Life Technologies (Grand Island, NY) and Rockland (Gilbertsville, PA), respectively. DMEM powder was purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum, L-glutamine, penicillin, and streptomycin were purchased from Life Technologies. CellTiter® 96 AQueous Assay and Nano-Glo® Luciferase assay system were purchased from Promega (Madison, WI).

Cell cultures and treatment

MN9D, a mouse-derived dopaminergic neuronal cell line, were maintained in DMEM media supplemented with 10% fetal bovine serum, 2mM L-glutamine, 50 units of penicillin, and 50μg/ml of streptomycin in a humidified incubator with 5% CO_2 at 37°C as described previously (Jin et al., 2011, Latchoumycandane et al., 2011). After exposing Mn9D cells to designated concentrations of Mn for 9

h, the cells were subjected to quantitative real-time RT-PCR or western blot analysis.

Plasmid constructs

Nucleotide sequence information of STAT5B promoter was obtained from NCBI. DNA from the brain of C57 black mice was isolated using DNeasy miniprep kit (Qiagen) and used as template to amplify the promoter region of STAT5B. In order to clone the promoter regions, primers were designed to bind to the specific location of promoters as depicted in the figures and contain restriction enzyme sequence either Nhe I or Kpn I with extra sequences. Primer sequences will be available upon request. The PCR amplicon were digested with proper restriction enzyme before ligated to pNL1.1 NanoLuc®Luciferase vector (Promega). All cloned promoter region of STAT5B were sequenced and confirmed. Site-directed mutageneses were performed to generate deletion mutant of putative transcription factor binding site. Briefly, mutagenic forward primers were designed to bind to the nucleotide sequence just after deletion site and amplified partial promoter region with reverse primer which bind to vector backbone. Similarly, mutagenic reverse primers were designed to bind to the nucleotide sequence just before deletion site and amplified partial promoter region with forward primer which bind to vector backbone. The resulting two PCR amplicons were used as template to generate full length promoter with two vector backbone primers. The final PCR amplicons were digested to proper restriction enzymes and ligated to pNL 1.1 Nano Luc®Luciferase vector. Each deletion mutant of STAT5B promoter 1 was sequenced and confirmed.

Promoter activity assay

Mn9D cells were transfected with luciferase reporter vector fused with either STAT5B promoter or empty vector together with lacZ vector 24h prior to exposure to Mn using lipofectamine2000 (Life Sciences). Treated cells were collected and lysed with lysis buffer (Promega), followed by luciferase assay (Promega) for the measurement of luciferase activity using Nano-glo®

Luciferase assay system (Promega). Cell lysates were further analyzed for β-galactosidase activity which was used for normalization.

Transfections

Mn9D cells were transfected with 10ug of promoter reporter vector or promoter reporter vector expressing STAT5B promoter with luciferase by using lipofectamine2000 reagent (Lifescience) according to manufacturer's protocol. Transfected cells were seeded in 12 well plates for promoter analysis, T-25 flasks for quantitative RT-PCR, or 96-well plates for cell proliferation assays before the treatment next day.

Cell proliferation assays

Cell survival was measured by colorimetric MTS assay (CellTiter® 96 AQueous Assay). Cells were seeded in 96-well plates before exposure to Mn. At the end of treatment, 20% MTS reagent was added to each well. The MTS tetrazolium compound is bioreduced in living cells to its colored formazan products. After incubating for up to an hour, absorbance at 490nm was measured using SpectraMax spectrophotometric plate reader (Molecular Devices).

Quantitative RT-PCR

After treatment, total RNA was extracted from cells or tissues using an Absolutely RNA Mini Extraction Kit (Agilent Technologies, Santa Clara, CA) according to manufacturer's protocol. Complementary DNAs were prepared with DNase I treatment using the High Capacity cDNA Synthesis Kit (Life Technology, Grand Island, NY).SYBR Green quantitative PCR was performed. Forward primer (5'- GCAATGATTATAGCGGCGAGA-3') and reverse primer (5'- CAAAGGCGTTGTCCCAGAGG-3') were used for detection of STAT5B mRNA. Primers for 18S RNA (Qiagen) were used as housekeeping gene control for normalization. The threshold cycle (C_t) was calculated from the instrument software, and fold change of gene expression for pair-wise comparisons was calculated using the $\Delta\Delta C_t$ method.

***In silico* analysis for putative transcription factors**

Putative regulatory promoter region were scanned to predict putative transcription factor binding sites with the Genomtix suit: http://www.genomatix.de/.

Statistical analysis

Data were analyzed using Prism 4.0 software (GraphPad Software, San Diego, CA). Paired Student *t*-test were performed. Tukey's multiple comparison test was used after ANOVA for comparing differences between multiple groups. Differences were considered statistically significant if p-values < 0.05 were obtained. Data are represented as the mean ± S.E.M. of at least two separate experiments performed at least in triplicate.

3. Results

3.1 Downregulation of STAT5B of mouse dopaminergic neurons upon Mn-induced neurotoxicity

We previously showed that Mn exposure compromised STAT5B expression to induce cytotoxicity in dopaminergic neuronal system (unpublished data). Mn was shown to reduce STAT5B expression at the protein level as well as the mRNA level. To study the effects of Mn on the transcriptional suppression on STAT5B gene expression in mouse dopaminergic neuronal cells, MN9D cells were used to examine whether Mn exposure induces downregulation of STAT5B. Mn exposure at 300 μM dose induced cytotoxicity. The cell viability of MN9D cells exposed to 300 μM Mn for 9 h was measured by performing MTS assay and showed that Mn exposure induced over 50% toxicity (Fig. 1B). The expression of STAT5B at the mRNA level after Mn exposure was measured by performing quantitative RT-PCR. The mRNA expression of STAT5B in MN9D cells treated with 300 μM Mn was

suppressed more than 40% compared to control, indicating Mn exposure significantly compromises STAT5B expression during Mn exposure (Fig. 1C).

3.2 Mn exposure suppresses promoter activity of STAT5B expression in dopaminergic neuronal cells

There are two alternatively spliced isoforms of STAT5B in the mouse, and both generate fully functional proteins. This alternative splicing was generated by the use of an alternative promoter and first exons with different sizes, respectively (Fig. 2). Although the promoter 2 generates an untranslated 519 bp long exon 1 (exon 1') and encodes the full length STAT5B protein, its usage is very limited and reported once in the thymus (http://fantom.gsc.riken.jp/3/db/annotate/%20main.cgi?masterid=E430022O15). Unlike promoter 2, promoter 1 of mouse STAT5B has been reported extensively and produces an untranslated 329bp long exon 1 (exon 1) (Fig. 2). Promoter 1 in mice shares high homology with human STAT5B. Hence, we decided to focus on the promoter 1 of mouse STAT5B to study how Mn exposure induces suppression STAT5B transcription. Genomic fragments of the mouse STAT5B promoter 1, from 2,000 bp upstream to 500 bp downstream, were cloned from C57 black mice as depicted in Fig. 3A and Fig. 4A. The genomic fragments of the promoter regions were fused to a Luciferase promoter reporter vector, pNL1.1.

To study Mn-induced suppression of the STAT5B promoter, we first analyzed the promoter activity of different 250 bp promoter regions by location (Fig. 3A). The 250 bp promoter fragments starting -2000 bp upstream of STAT5B exon1 to +500 bp downstream were cloned into the pNL1.1 as depicted in Fig 3A. The genomic fragments of the promoter

were sequenced and validated with the information of the nucleotide sequences of the mouse STAT5B promoter 1 obtained from NCBI webpage. Primers were designed to bind to each promoter fragment Promoter-reporter systems were transfected into MN9D cells containing either empty vector or the STAT5B promoter fragments, followed by exposure to 300μM Mn for 9 h. The proximal promoter showed stronger transcriptional activity compared to the distal promoter region of the STAT5B promoter in both control cells and Mn exposed cells. Specifically, the promoter region from 250 bp upstream to 500 bp downstream of exon 1 showed strong transcriptional activity, whereas the region between -500 bp upstream to -250 bp showed less promoter activity. However, proximal regions between 250 bp upstream to 250 bp downstream showed strong repression of the promoter during exposure to Mn. Next, we examined whether Mn exposure induces synergistic effects in suppressing promoter activity in transcription of STAT5B. We subcloned a series of various promoter regions of different length (Fig. 4A). The longer region of the promoter did not strengthen promoter activity compared to the shorter proximal region of promoter (Fig. 4B). Inclusion of the 500 bp upstream to 250 bp upstream region greatly repressed promoter activity. As the genomic fragment from 500 bp to 250 bp upstream region demonstrated highly repressed promoter activity and addition of the region into the longer genomic regions from 500 bp upstream to 500 bp downstream repressed the promoter activity, this region may have repressive elements controlling the expression of STAT5B. Addition of the upstream region of the promoter, above 500 bp upstream, did not further reduce promoter activity which also supports the hypothesis that the genomic fragment from 500 bp upstream to 250 bp upstream may contain transcription repressive element. The longer distal promoter regions from 2000

bp upstream to 1000 bp upstream did not appear to have strong promoter activity of STAT5B transcription. Mn exposure repressed the promoter activity in most promoter regions. However, it was the promoter region from exon 1 to 00 bp downstream that showed strong repressive effects by Mn exposure compared to control. Addition of all other regions did not induce additive repressive effects in promoter activity. These results suggest that the promote regions from exon 1 to 500 bp downstream may have accumulative effects in repression of promoter activity, implying the Mn exposure-responsive and sensitive repressive elements may exist in this region. Interestingly, the region containing strong general repressive element did not show repressive effects by Mn exposure, suggesting this region may be activated by other mechanisms.

3.3 Repressor responds to Mn exposure to suppress STAT5B transcription

To identify putative transcription factors that bind to the STAT5B promoter, we used genomatix suit software to perform *in silico* analysis of the proximal region from 250 bp upstream to 500 bp downstream of exon 1, which appears to have Mn exposure-responsive elements. Among the *in silico* potential transcription factors, only transcriptions factors which have been reported to bind to STAT5B and those that have scores higher than 0.95 were selected (Fig 5). The binding site of each potential transcription factor was deleted via site-directed mutagenesis in order to validate whether the putative transcription factors were able to bind to the selected region of STAT5B promoter 1 and respond to Mn exposure to regulate the expression of STAT5B. The promoter regions were fused to a luciferase reporter system containing STAT5B promoter 1 with either a mutant binding site or wild type and

then were transfected into MN9D cells before exposure to 300 µM Mn. We found that mouse KLF and KLF7 play a role in STAT5B transcriptional repression in normal condition (Fig. 6 A and C). A mouse KLF binding site is located within exon 1, and deletion of this binding site in the promoter induced stronger transcription activity, indicating that this factor may play a role as a transcription repressor (Fig. 6 A). Surprisingly, 300µM Mn exposure failed to repress transcriptional activation of the STAT5B promoter 1. Another putative transcription factor is SP1 and an SP1 binding site is present within exon 1 (Fig 6. B). Deletion of this SP1 binding site significantly reduced transcriptional activity, to roughly 50% in the absence of Mn exposure compared to wild type control, indicating SP1 may play a role as a transcriptional activator. Mn exposure of 300 µM further reduced transcriptional activity from the already reduced transcriptional activity due to the deletion of binding site of SP1. However, the reduction of transcription activity of the deletion mutant over the control in the absence of Mn exposure was less than the reduction of transcriptional activity in wild type. This may imply that the role of SP1 as a potential transcriptional activator may be reduced in the presence of Mn exposure. Another putative transcription factor located in intron 1 is KLF7 (Fig 6. C). Deletion of the KLF7 binding site did not affect the transcriptional activity on STAT5B promoter 1 in the absence of Mn exposure. However, when neuronal cells were exposed to Mn, KLF7 deletion mutant showed higher activity than the transcription activity of wild type. These results indicate that two KLF7 transcription factors may sense the Mn exposure and work as transcription repressors in the presence of Mn exposure. Sp1 transcription factor binding to the promoter region in exon 1 may also have a role as

transcription activator. This Sp1 transcription factor may become less active during Mn exposure.

4. Discussion

Overexposure of Mn in neurons results in cytotoxic cell death leading to the development of neurodegenerative diseases that shows similar clinical symptoms like that of PD (Bowman et al., 2011, Caudle et al., 2012, Farina et al., 2013). It has been known that STAT5B potentially facilitates cell growth and also protects the cells against multiple stress scenarios and toxicity (Du et al., Buitenhuis et al., 2004, Paukku and Silvennoinen, 2004, Tan and Nevalainen, 2008, Han et al., 2009, Cholez et al., 2012, Kalita et al., 2013, Abroun et al., 2015). We have recently observed that there was significant downregulation of STAT5B in the neurons post exposure to Mn (unpublished data). This present study provides a vivid evidence on the down regulatory effects of Mn exposure on STAT5B by modulation of transcriptional factors in neurons.

For understanding the mechanisms of Mn-induced downregulation of STAT5B, an *in vitro* neuronal cell culture model was employed using the Mn9D mouse dopaminergic neuronal cells.. Exposure of Mn9D neuronal cells to 300 μM Mn for 9 h induced a neurotoxic cell death.(Fig. 1A). It was also observed that STAT5B mRNA expression was downregulated in MN9D cells post the Mn exposure (Fig. 1B). These results coherently consistent with our recent findings on the N27 rat dopaminergic neuronal cells, showing downregulation of STAT5B prior to exposure with 300 μM Mn (unpublished data).

The mouse model commonly expresses two alternative splicing forms of STAT5B with two different promoters. Major splice form of STAT5B uses promoter 1 which was identified and reported in multiple organs (Fig. 2). Whereas the promoter 2 corresponding to the mouse STAT5B has been reported by a Japanese group only once in the thymus tissue (unpublished data). Interestingly it has been shown earlier that promoter 1 of STAT5B shares a high homology between human, mouse and rat(Crispi et al., 2004). Henceforth we have employed the mouse STAT5B for our studies.

The promoter studies performed at different locations using 250 bp genomic fragments of STAT5B promoter showed that the proximal location of the promoter contains strong promoter activity (Fig. 3). Moreover, the proximal promoter region indicated the strongest promoter activity, confirming that transcription activators may bind in the proximal region near to exon 1. Also, a sudden reduction of promoter activity was observed at 500 bp upstream indicating that the 500 bp upstream region might contain repressor activity. Mn exposure suppressed the transcriptional STAT5B expression throughout location of promoters. This in addition may indicate that Mn exposure might be involved in suppression of overall transcriptional activity of STAT5B in a wide range of promoter regions. However, proximal promoters near exon 1 were shown to be more sensitive to Mn exposure in reduction of transcriptional activities.

Differential length of STAT5B promoter was examined to study whether Mn exposure induces synergistic effects in suppressing transcription of STAT5B (Fig. 4). Surprisingly, the transcriptional activities did not show additive effects in activating transcription. Whereas, the combining of proximal promoter regions from 500 upstream to

500 downstream showed less transcriptional activity compared to 250 bp downstream promoter region. Alternatively, it could be interpreted that transcription repressor regulated the transcription of STAT5B. Further analysis of longer fragments of STAT5B promoter showed lesser transcriptional activity. As a result, we were driven to suggest that multiple transcription repressors when compared to longer range of promoter region might be involved in regulation of transcriptional activation and repression in the expression of STAT5B. Note-worthily, promoter region from exon 1 to 500 bp downstream is revealed to have an accumulative effect in repressing promoter activities during Mn exposure. This repressive effect of promoter region 500 upstream to 250 upstream did not limit further from basal repressive effects. These cumulative results suggest that the transcription factorss that can sense Mn exposure and regulate STAT5B transcription might be present in exon 1 and in the downstream region.

The *in silico* analyses performed with Genomatix suites showed the putative binding of TFs to the promoter 1. TATA and CAAT box were not found in the promoter 1, whereas exon 1 contained a high GC content and many more TF binding sites such as zinc finger TF and SP1 binding sites. Human STAT5B promoter does not have a TATA and also a CAAT box but contains multiple SP1 sites (Crispi et al., 2004). Thus it is possible that the mechanisms in response to Mn exposure to regulate mouse STAT5B expression, may also apply for humans . As seen from our results the mouse KLF binds to the promoter region at 41 bp of exon 1 (Fig. 6 A). Site-directed deletion mutant of this binding site of mouse KLF does not affect basal transcriptional activity in the absence of Mn. However, the transcriptional activity of this deletion mutant was almost not repressed during Mn exposure,

thereby indicating that the mouse KLF suppresses transcription only in the presence of Mn. Surprisingly, another putative TF binding site for KLF7, was present in the downstream of exon 1 (Fig. 6 C). Like as in the mouse KLF, the deletion of this binding site for KLF7 did not harm the basal transcriptional activity. However, deletion of KLF7 binding sites compromised the reduction post Mn exposure. Interestingly, one SP1 present in exon 1 functioning as a transcriptional activator was identified to be sensitive to Mn treatment which further induced reduction of transcriptional activation with Mn treatment. It was not very surprising that SP1 was sensitive to Mn exposure and also that it regulates the expression of STAT5B. This is because SP1 has been shown previously to be sensitive and also that it gets activated during Mn exposure to induce upregulation of caspase-3 transcription (Uchida et al., 2012). It has also been reported that Sp1 acts as transcription repressor with histone deacetylase (Kaczynski et al., 2001). Collectively these data suggest that Mn exposure alters activities of TF to regulate STAT5B transcription.

In conclusion, this report demonstrates that Mn exposure reduced STAT5B expression at the mRNA level and alters the activities of TFs to downregulate transcription of STAT5B in dopaminergic neuronal cells. Also interestingly, Sp1 acts as a transcription activator sensitive to Mn exposure, whereas KLF plays the function as a transcription repressor.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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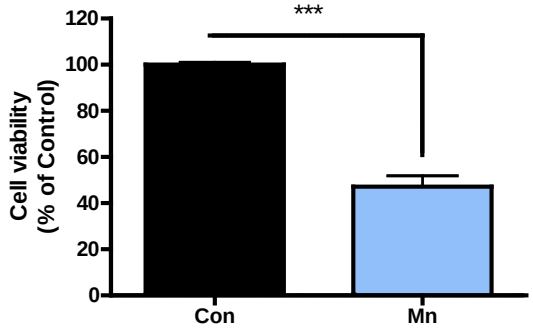
A)



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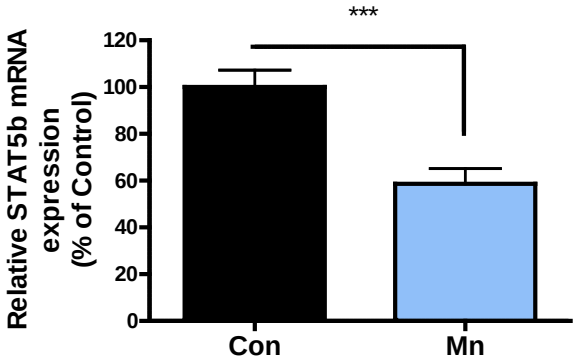

Fig. 1. Manganese (Mn) induces downregulation of STAT5B in dopaminergic neuronal cells. Mouse dopaminergic neuronal cells, Mn9D, were exposed to 300μM Mn for 9h. MTS assay was performed to measure Mn induced cytotoxicity (A). Quantitative expression of STAT5B was measured at mRNA level post exposure to Mn (C). Asterics *p<0.05 for control versus Mn-treated group. The results represent the mean S. E. of three individual replicates of experiments.

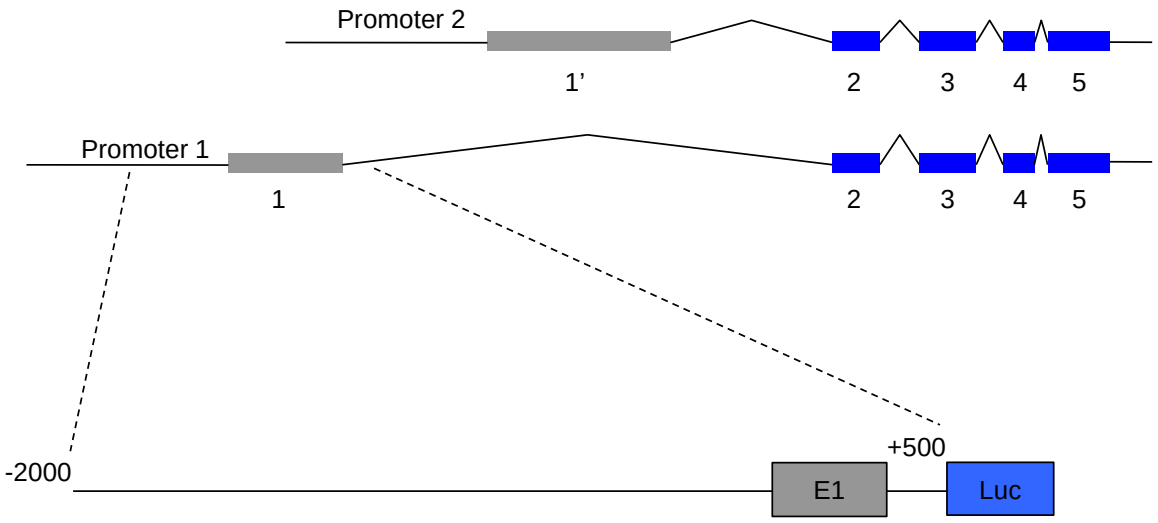

Fig. 2. Schematic presentation of mouse STAT5B promoter. Translated exons were represented with blue box, whereas untranslated exon was represented with grey box. Introns were represented with line. STAT5B promoter 1 region from 2,000 bp upstream to 500nt downstream was cloned for further promoter analyses. Subcloned STAT5B promoter was fused to Luciferase reporter system.

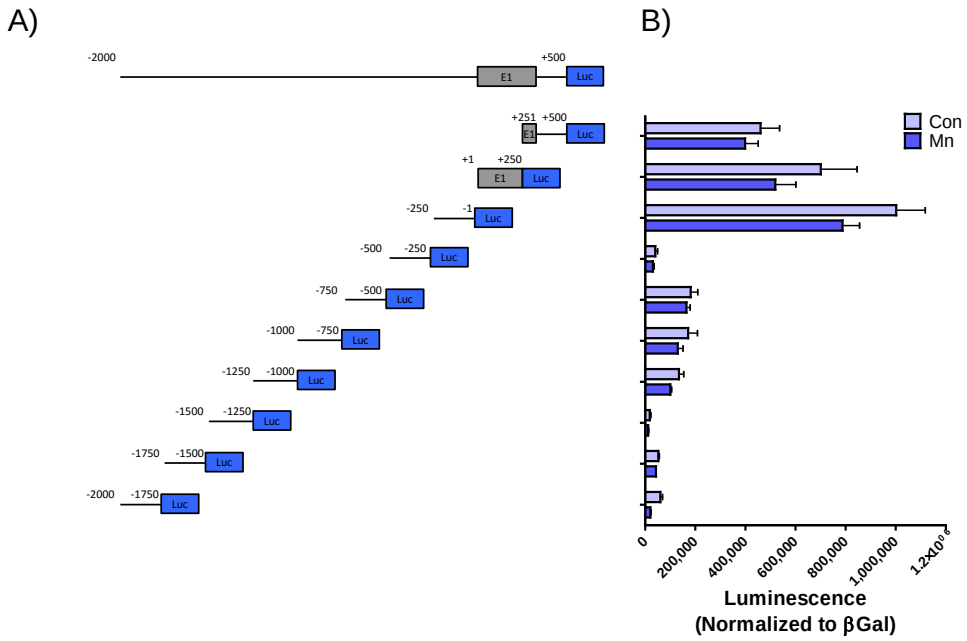

Fig. 3. Analyses of STAT5B promoter 1 in different location during Mn exposure. STAT5B promoter 1 regions with 500 bp was subcloned and subsequently fused to luciferase reporter system (A). STAT5B Promoters fused to luciferase reporter system were transfected to Mn9D dopaminergic neuronal cells. After 300 μM Mn exposure for 9h, promoter activities were measured via luminescence (B). Asterics *$p<0.05$ for control versus Mn-treated group. The results represent the mean S. E. of three individual replicates of experiments.

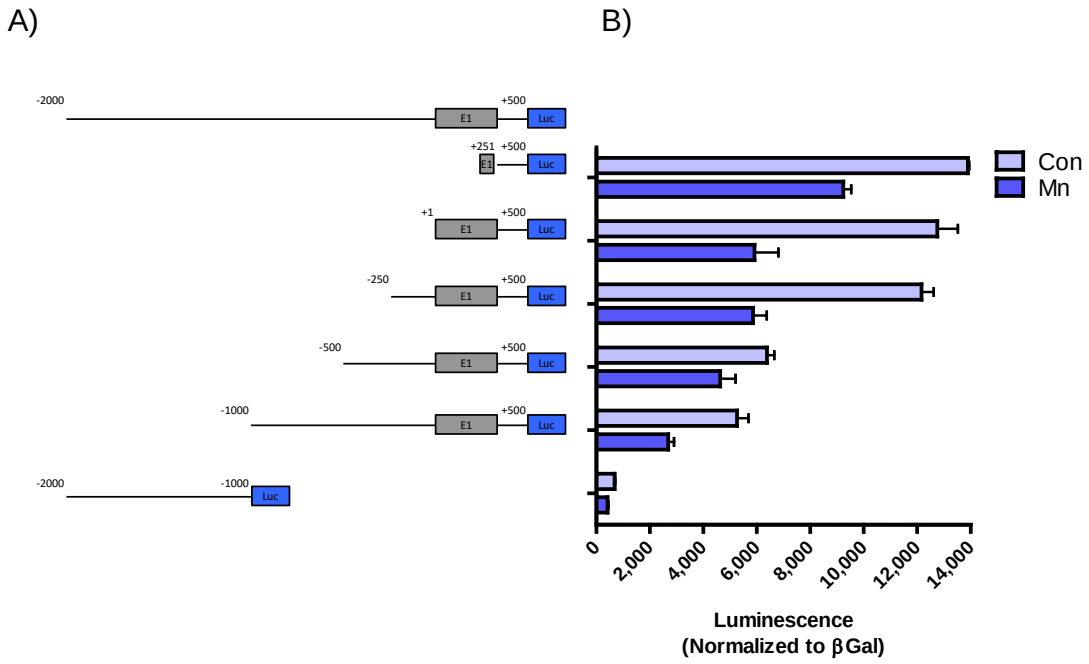

Fig. 4. Analyses of diffential length of STAT5B promoter 1 during Mn exposure. STAT5B promoter 1 was subcloned in differential length from downstream of exon 1 (A). STAT5B Promoters fused to luciferase reporter system were transfected to Mn9D dopaminergic neuronal cells. After 300 μM Mn exposure for 9h, promoter activities were measured via luminescence (B). Asterics *$p<0.05$ for control versus Mn-treated group. The results represent the mean S. E. of three individual replicates of experiments.

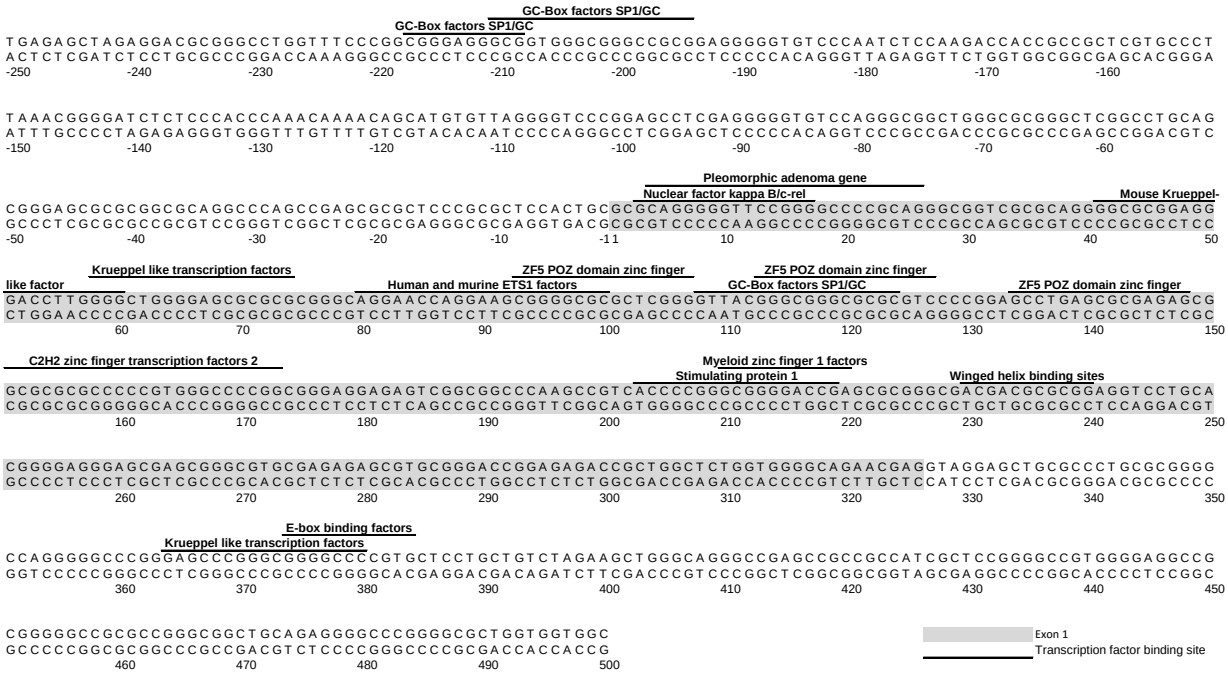

Fig. 5. *In silico* analysys of potential transcription factors binding to proximal region of STAT5B promoter 1. Potential transcription factors of STAT5B promoter 1 from 250 bp upstream to 500 bp downstream encompassing exon 1 were searched through MatInspector from Genomatix suites. Transcription factors either with experimental evidence or high score over 0.95 were shown.

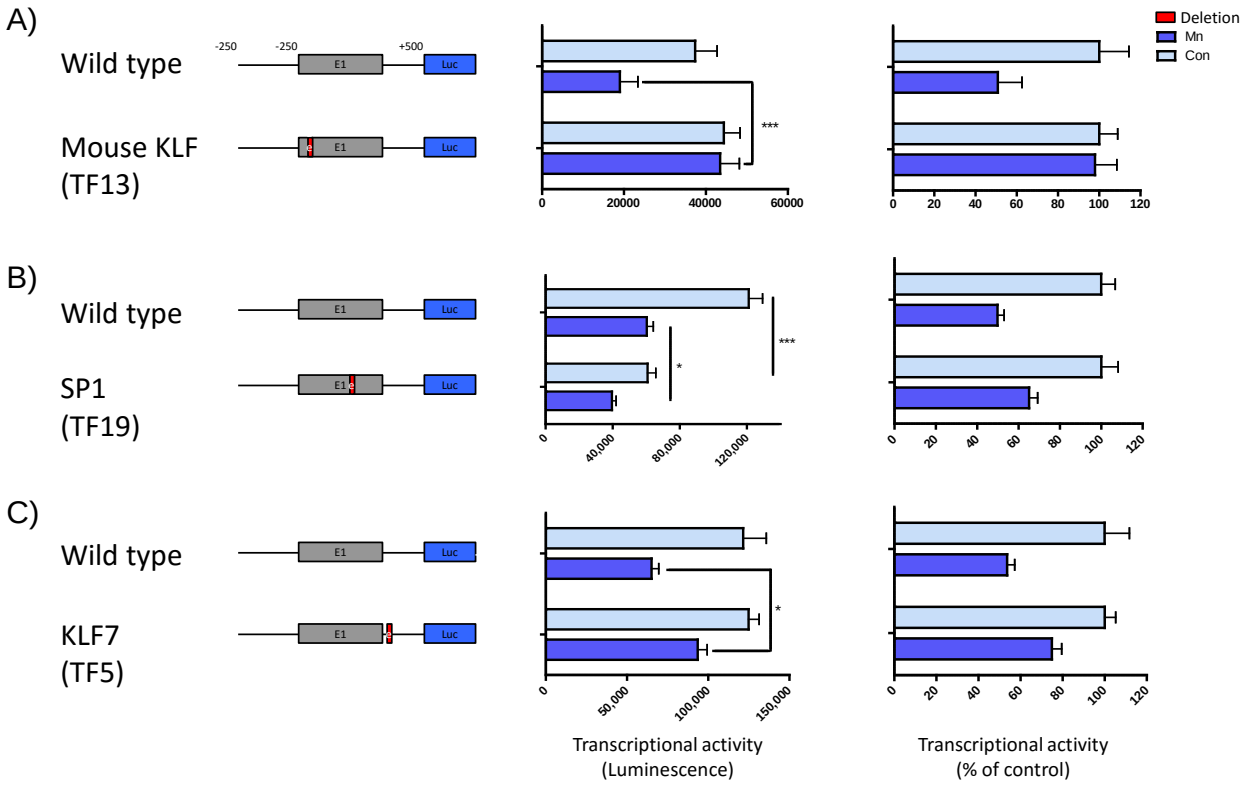

Fig. 6. Transcription factors repress transcriptional expression of STAT5B during Mn exposure. Transcription factor binding sites were deleted via site-directed mutagenesis (left panels of A, B and C). Red box denotes the sites of deletion of TF binding sites. Either wild type or deletion mutant of transcription factor binding sites were transfected to Mn9D cells 1day before exposed to 300 μM Mn for 9h. Promoter activities were measured via luminescence (middle panels of A, B and C). Promoter activities of either wild type or deletion mutant were compared to control group (% of control). Asterics ***$p<0.001$ and *$p<0.05$ for control versus Mn-treated group. The results represent the mean S. E. of three individual replicates of experiments.

CHAPTER V: GENERAL CONCLUSIONS

This section presents an overview of the results and findings of this dissertation, with a special emphasis on overall implications of these findings for developing novel neuroprotective strategies for Parkinson's disease. The major findings of each research chapter included in this dissertation are covered in the discussion section of the relevant chapter.

p73 gene is highly susceptible to Mn induced neurotoxicity in Dopaminergic Neurons

The major finding from Chapter 2 of the thesis is that we identified the p73 genes which is sensitive to Mn exposure and play an important role in Mn-induced neurotoxicity in nigrostriatal system. To our knowledge this finding is the first evidence that Mn exerts its neurotoxic effects by suppressing the expression of the anti-apptotic ΔNp73 protein. A few of key molecules that can mediate Mn exposure to dopaminergic neuronal cell death have been identified including PKCd, Caspase-3, Caspase-9, YY1, and p53 (Anantharam et al., 2004, Latchoumycandane et al., 2005b, Yang et al., 2007, Zhang et al., 2007b, Jin et al., 2011, Jin et al., 2014, Karki et al., 2014, Wan et al., 2014, Ma et al., 2015). Despite of the breakthrough findings of these key molecules, the exact molecular and cellular mechanisms underlying Mn-induced neurotoxicity remain elusive. The analyses of the expression of 84 apoptotic genes in SN of mice treated with 10 mg/kg for 30 days revealed several key molecules such as Bok, TNFRSF1a, and p73. The p73 protein was shown to play important roles in a wide variety of cellular events including cell death, self-renewal of neural stem cells, cell cycle regulation, differentiation of neurons, surveillance of damaged (Yang et al.,

2002, Ozaki et al., 2005, Killick et al., 2011, Grespi and Melino, 2012, Di et al., 2013, Jancalek, 2014, Engelmann et al., 2015, Sabapathy, 2015). p73 gene was shown to be implicated in neuronal development, tumor, and neurodegeneration (Grespi and Melino, 2012, Jancalek, 2014). Two classes of p73 protein producs, TAp73 containing transactivation domain and ΔNp73 lacking transactivation domain, antagonize each other. From the following *in vivo* experiment in which C57 black mice were orally exposed to 30 mg/kg for 30 days via gavage, the results of PCR array system were validated. Biochemical analyses of SN revealed ΔNp73 was downregulated in Mn induced neurotoxicity. Further characterization of p73 in Mn-induced neurotoxicity was performed using N27 dopaminergic neuronal cells. The *in vitro* studies showed consistent results that ΔNp73 was downregulated in dopaminergic neuronal cell system. The downregulation of ΔNp73 was shown to be time-dependent and dose-dependent manner in N27 cells and mouse primary striatal cells. The results of downregulation of Bcl-xL and Mcl-1, downstream effector molecules of p73 pathway, indicated that Mn exposure suppresses ΔNp73 pathway. We also showed that downregulation of ΔNp73 is independent of activated Caspase-3, as pre-treatment of z-DEVD-fmk failed to rescue ΔNp73 from Mn-induced neurotoxicity. Finally, we showed that ΔNp73 was anti-apoptotic against Mn-induced neurotoxicity. We overexpressed ΔNp73 in N27 dopaminergic neuronal cells and performed cell viability assay which revealed that ΔNp73 can protect N27 cells from Mn-induced neurotoxicity at lower Mn dose ranging 100 to 200 μM. Taken together, we demonstrated that ΔNp73 is anti-apoptotic and was compromised during Mn exposure. These observations provide new insights into the mechanisms underlying Mn-induced dopaminergic neurotoxicity.

Mn exposure downregulates neuroprotective STAT5B in dopaminergic neurons

The major findings in Chapter 3 were the identification of another key molecule, STAT5B, that plays a neuroprotective role in dopaminergic neuronal system during Mn exposure. This finding came across in the course of identifying the downstream targets of PKCd in Mn-induced neurotoxicity. Protein PKC delta was previously shown in our laboratory to play important roles in Mn-induced neurotoxicity (Kitazawa et al., 2005, Latchoumycandane et al., 2005b, Zhang et al., 2007b, Afeseh Ngwa et al., 2011, Jin et al., 2014). Interestingly, we found that expression of STAT1 was not greatly altered during Mn exposure, suggesting that Mn exposure selectively induces downregulation of STAT5B. We characterized the downregulation of STAT5B in Mn-induced neurotoxicity. STAT5B was downregulated in time and dose dependent during Mn exposure. Mouse dopaminergic neuronal cell, MN9D, and mouse primary striatal culture also showed downregulation of STAT5B. STAT5a was shown to be sensitive to proteasomal degradation pathway (Wang et al., 2000). We used lactacystin, an inhibitor to proteasomal degradation pathway, to answer whether STAT5B may be degraded via proteasomal degradation pathway. The results from the studies with lactacystin pre-treatment showed that downregulation of STAT5B from Mn-induced neurotoxicity was independent of proteasomal degradation pathway. The plausible mechanism behind downregulation of STAT5B came from the studies with anti-oxidant drug, which showed that pre-treatment of NAC prevented downregulation of STAT5B against Mn-induced neurotoxicity. MPP^+ is neuotoxicant that selectively inhibit dopaminergic neurons and generate excessive ROS. Exposure to MPP^+ also reduced STAT5B expression, supporting the hypothesis that Mn exposure induces excessive oxidative stress which in turn

downregulate STAT5B expression. We also characterized the role of STAT5B in Mn-induced neurotoxicity in dopaminergic neurons and found that STAT5B plays a role of protecting the neurons against Mn exposure. We overexpressed STAT5B in N27 cells and challenged Mn exposure. The results showed that overexpression of STAT5B protected N27 cells against Mn exposure. The findings that Mn exposure induces downregulation of STAT5B were also encapitulated in *in vivo* animal model. C57 black mice were exposed to 30 mg/kg Mn for 30 days. The expression of STAT5B was downregulated in SN of the Mn treated C57 black mice compared to the control group. We further examined the MitoPark PD mouse model. We found that STAT5B expression was downregulated in SN of 25wk old MitoPark mice. These data are novel findings and provide potential drug candidate for neurodegenerative disease which involves oxidative stress. We show that Mn exposure downregulates STAT5B expression in human lymphocytes. Taken together, our results suggest that Mn exposure downregulates STAT5B in striatal neurons and that Mn-induced downregulation of STAT5B may compromise the protective signaling pathway, thereby exacerbating neuronal cell death.

Mn exposure alters profiles of transcription factors to suppress transcription of STAT5B in dopaminergic neurons

In the Chapter 4 we extended mechanistic studies of novel findings from Chapter 3. Major findings in chapter 4 were that Mn exposure alters profiles of transcription factors to suppress transcription of STAT5B. We subcloned the promoter region of mouse STAT5B from 2,000 nt upstream to 500 nt downstream. We subcloned a series of STAT5B promoter

regions in different location and differential length of STAT5B promoter regions. From the promoter analysis of different location we showed that proximal promoter region of STAT5B contained strong transcription activities. These proximal promoter regions were shown to contain elements that respond to Mn exposure to repress transcription of STAT5B. Promoter studies of differential length of STAT5B promoter region showed that promoter region containing exon 1 and proximal downstream region of promoter can synergize the repressive effects in repressing the transcription upon Mn exposure. However, the repressive effects from distal promoter region was not synergistic to the effects of proximal promoter regions of STAT5B. We provided putative transcription factors binding to promoter region from

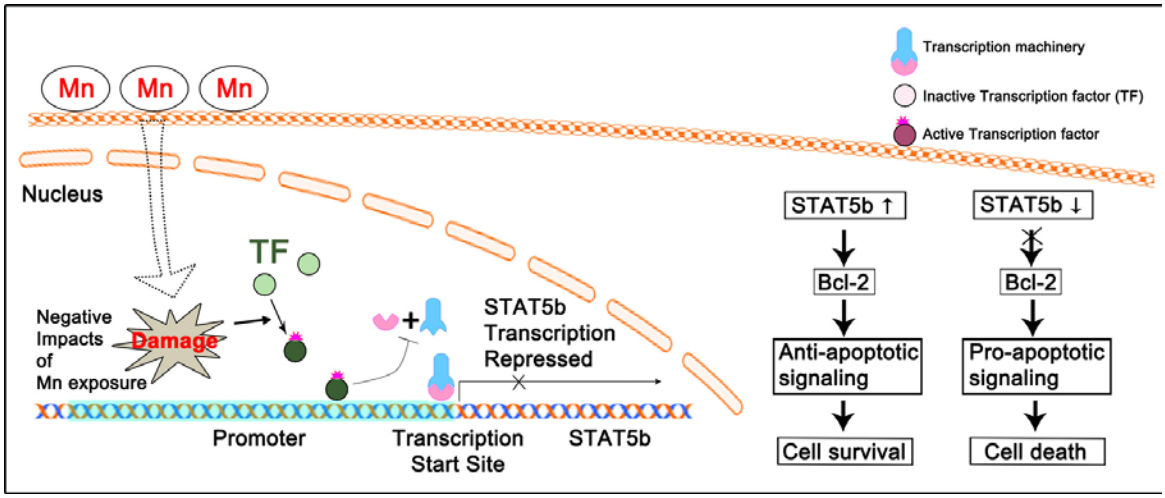

Figure 1. Schematic view of suppression of transcription of STAT5B during Mn exposure

exon 1 to 250 nt of downstream region via *in silico* analysis. Several Sp-1 like factor binding sites were identified. Site-deletion mutation of transcription factor binding sites showed that two KLFs function as transcription repressor that sense the Mn exposure and activated upon Mn exposure. In addition, one Sp1 transcription factor binding site was shown to reduce transcription activation during Mn exposure. Taken together, these data suggest Mn exposure

alters the profiles of transcription factors to downregulate anti-apoptotic STAT5B signaling via Sp1-like transcription factor-dependent mechanism in dopaminergic neurons, which may significantly contribute to Mn neurotoxicity.

In summary, we have identified two neuroprotective genes that are susceptible to Mn exposure. Mn exposure suppressed expression of ΔNp73 and STAT5B to exert its neurotoxicity. Mn exposure also alters transcription of STAT5B via transcription factors. These novel findings may provide important insight in understanding Mn-induced.

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