Thesis title

The mechanisms of protective function of DJ-1 in Parkinson's models of neuronal loss: VHL and PON2

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Abstract of thesis entitled

The mechanisms of protective function of DJ-1 in Parkinson's models of neuronal loss: VHL and PON2

Parkinson's disease (PD) is the most common neurodegenerative motor disorder, whose clinical features are rest tremor, bradykinesia, muscular rigidity and postural instability. Although most reported cases are sporadic, a handful of familial cases and their causative genes have been identified. Loss-of-function mutations in DJ-1, one of these genes, are responsible for 1% of familial PD cases. Our laboratory has previously reported that DJ-1-lacking neurons are sensitive to oxidative stress, induced by hydrogen peroxide or the neurotoxin MPTP. To investigate the possible mechanisms through which DJ-1 protects against oxidative stress, we performed a proteomic screen and identified Von Hippel Lindau (VHL) and Paraoxonase2 (PON2) as potential DJ-1 interacting partners. VHL is an E3 ubiquitin ligase which, in normal conditions, poly-ubiquitinates HIF-1α, a subunit of a master hypoxic/oxidative stress transcription factor, whose function is protective in oxidative and hypoxic stresses. In the present study, we provided further evidence of interaction of DJ-1 with VHL. We also demonstrated that HIF-1α protein level, as an indicator of VHL activity, is lower in cells lacking DJ-1, suggesting the inhibitory role of DJ-1 on VHL. Our *in vitro* studies also showed that DJ-1 inhibits ubiquitin ligase activity of VHL on HIF-1α by reducing the VHL-HIF-1α interaction. Importantly, accumulation of HIF-1 protects embryonic cortical neurons against MPP+ induced neuronal death. Finally, we confirmed the impairment of HIF-1 response to oxidative stress in human lymphoblastoids of DJ-1-linked PD cases. In the second part of this study, we demonstrated the interaction of DJ-1 and PON2. Interestingly, PON2 lactonase activity is reduced in DJ-1

deficient cells which could be rescued by re-introduction of DJ-1, suggesting a modulating role of DJ-1 on PON2 activity. In addition, PON2 deficiency, like DJ-1 deficiency, hypersensitizes neurons to MPP+, which could be rescued by over-expression of PON2 in both cases. Taken together, our data provide evidence that DJ-1 exerts its protective role by inhibiting VHL activity, enhancing HIF-1α stability, and increasing PON2 pro-survival function in PD models.

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List of Manuscripts

I. DJ-1 interacts with and regulates PON2, an enzyme critical for neuronal survival in response to oxidative stress.

Parsanejad M, Bourquard M, Zhang Y, Rousseaux M.W, Qu D, Aleyasin H, Irrcher I, Callaghan S, Vaillant D.C, Kim R.H, Slack R.S, Mak T.W, Reddy S.T, Figeys D and Park D.S.

The manuscript has been submitted to the Journal of Neurochemistry.

II. Regulation of the VHL/HIF-1 pathway by DJ-1

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Appended Articles:

I. DJ-1 protects the nigrostriatal axis from the neurotoxin MPTP by modulation of the AKT pathway.

Aleyasin H, Rousseaux MW, Marcogliese PC, Hewitt SJ, Irrcher I, Joselin AP, **Parsanejad M**, Kim RH, Rizzu P, Callaghan SM, Slack RS, Mak TW, Park DS.

Proceeding of the National Academy of Science USA. 2010 Feb 16;107(7):3186-91. Epub 2010 Jan 26.

II. Pim-1 kinase as activator of the cell cycle pathway in neuronal death induced by DNA damage.

Zhang Y, **Parsanejad M**, Huang E, Qu D, Aleyasin H, Rousseaux MW, Gonzalez YR, Cregan SP, Slack RS, Park DS.

J Neurochem. 2010 Jan;112(2):497-510. Epub 2009 Nov 6

III. Role of Cdk5-mediated phosphorylation of Prx2 in MPTP toxicity and Parkinson's disease.

Qu D, Rashidian J, Mount MP, Aleyasin H, **Parsanejad M**, Lira A, Haque E, Zhang Y, Callaghan S, Daigle M, Rousseaux MW, Slack RS, Albert PR, Vincent I, Woulfe JM, Park DS.

Neuron. 2007 Jul 5;55(1):37-52.

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List of Abbreviations

ALS= Amyotrophic lateral sclerosis

AMPA= α-amino-3-hydroxy-5-methylisoxazole proprionic acid

ASIC= acid sensing ion channel

ATP = Adenosine Triphosphate

1BnTIQ= 1-benzyl-1,2,3,4-tetrahydroisoquinoline

ccRCC= clear-cell renal cell carcinoma

CMA= chaperon-mediated autophagy

CNS= Central nervous system

COMT= Cattechol-*O*-methyl transferase

COR= C-terminal of Ras domain (in LRRK2)

COX= cyclooxygenase

CSF= cerebrospinal fluid

CT = computed tomography

DA= Dopamine

DBS= Deep brain stimulation

DGAT1= diacylglycerol acyltransferase1

DHB= 3,4-dihydroxybenzoate

DLB= dementia with Lewy body

L-DOPA= L-3,4-dihydroxyphenylalanine

eIF2α= eukaryotic translation initiation factor 2 α

EPO= erythropoietin

ER= Endoplasmic reticulum

ETC= electron transport chain

Gpe= external globus pallidus

Gpi= internal globus pallidus

GPx= Glutathione peroxidase

GSH= reduced glutathione

HDL= High density lipoprotein

HIF-1= hypoxia inducible factor 1

HNE= 4-hydroxy-2-nonenal

HRE= hypoxia-responsive element

IBR= in-between ring

ILs= interleukins

iNOS= inducible nitric oxide synthase

JNK= c-Jun N-terminal kinase

KO= knock-out

LDL= Low density lipoprotein

LOX= lipoxygenase

LPS= lipopolysaccharide

LRRK2= Leucine rich repeat kinase 2

MAO= monoamine oxidase

MAPK= mitogen-activated protein kinase

MDA= malondialdehyde

MEF= murine embryonic fibroblasts

MHC= major histocompatibility complex

MKK= MAP kinase kinase

MPP^+= 1-methyl-4-phenylpyridinium

MPM= mouse peritoneal macrophages

MPTP= 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MRI = magnetic resonance imaging

mtDNA= Mitochondrial DNA

NADH= Nicotinamide adenine dinucleotide (reduced)

NMDA= N-methyl-D-aspartate

Nrf2= Nuclear factor erythroid 2-like 2

ODD= oxygen dependent degradation

8-OH-G= 8-hydroxyguanosine

PD= Parkinson's disease

PET= Positron emission tomography

PGC-1α= peroxisome proliferator-activated receptor gamma coactivator-1a

PHD= prolyl hydroxylases

PI3K= phosphatidylinositol-3-kinase

PINK1= PTEN induced putative kinase 1

PON1,2,3= Paraoxonase1,2,3

PTP= permeability transmembrane pores

RCC= Renal cell carcinoma ()

REM = Rapid eye movement

RNS= reactive nitrogen species

ROC= Ras of complex (in LRRK2)

ROS= Reactive oxygen species

RT-PCR= Reverse-transcriptase polymerase chain reaction

SNc= Substantia nigra pars compacta

SNCA= Alpha synuclein (human gene)

SOD= Superoxide dismutase

SPECT= Single photon emission computed tomography

SSRIs= selective serotonin reuptake inhibitors

STN= subthalamic nucleus

TFAM= mitochondrial transcription factor A

TNF-**α=** tumor necrosis factor-**α**

UPR= unfolded protein response

VEGF= vascular endothelial growth factor

VHL= Von Hipple Linau

CHAPTER 1

General Introduction

1.1 Parkinson's Disease

Parkinson's disease (PD), first described by and named after British surgeon James Parkinson in 1817, is the second most common neurodegenerative disease, exceeded in frequency only by Alzheimer's disease. PD is a chronic and progressive disease characterized by a set of neurological syndromes termed Parkinsonism, including resting tremor, rigidity, bradykinesia (or akinesia in severe cases), and postural disturbance. According to National Institute of Health (NIH) data, PD affects over 1 million people in North America and over 4 million people worldwide. This devastating neurological disease imposes a social and financial load on both developed and developing countries and poses a huge challenge to biomedical research.

1.2 Epidemiology

In industrialized countries the frequency of PD is approximately 0.3% of the whole population, around 1% of people over 60 years of age (Nussbaum and Ellis, 2003) and up to 4% of people over 80 years old (reviewed in (de Lau and Breteler, 2006)). According to the Parkinson's Society of Canada (www.parkinson.ca), PD affects approximately 100,000 people in Canada and its incidence rate is 10-20 per 100,000.; nearly 300-600 Canadian patients are newly diagnosed every year. It is difficult to estimate the world wide incidence rate as there is a significant conflict between the results of different reports. For instance, in Europe, the incidence of PD ranges from 65.6 to 12,500 per 100,000, based on 39 studies (reviewed by (Wattel et al., 1990; Zhang and Roman, 1993; von Campenhausen et al., 2005; Muangpaisan et al., 2009; Wright Willis et al., 2010)). PD patients' life expectancy is markedly reduced compared to the control population (Lang and Lozano, 1998), and quality

of life is reduced. As the disease progresses, severely disabled patients become progressively unable to take care of themselves, imposing a drastic burden on their families and healthcare workers. The impacts of the medical costs of PD on families and care givers can also be overwhelming. For instance, it puts a financial burden of approximately $558.1 million per year on the health care system in Canada (Source: Parkinson's Society of Canada www.parkinson.ca).

1.3 Clinical manifestation

1.3.1 Motor symptoms. PD appears as both a motor and a non-motor disease. Its classical motor symptoms are:

Tremor. Although this is the most common motor symptom of PD, 30% of cases do not show this feature at the beginning of the disease (Jankovic, 2008). Tremor usually shows up at rest and disappears with voluntary movements (Jankovic, 2008). It is mostly unilateral at the onset of PD and bilateral at more advanced stages of the disease (Jankovic, 2008).

Muscular rigidity. Rigidity or stiffness of muscles is basically due to increased muscle tone (Jankovic, 2008). This affects neck and shoulder at the beginning and spreads to the whole body during the progression of the disease.

Slowness of movement (Bradykinesia). This is considered the most incapacitating motor symptom of early stages of PD (Samii et al., 2004), and involves impairments of planning, initiating and executing movement (Berardelli et al., 2001). This causes difficulties in motor activities which need subtle motor control (such as writing and buttoning), spontaneous movements, swallowing, and arm swinging.

Postural instability. This is caused by impairment of postural reflexes and leads to impaired balance. Postural instability typically occurs in late stages of PD (Jankovic, 2008).

1.3.2 Nonmotor symptoms. Traditionally, PD is known as a motor disorder. However, its symptoms are not limited to motor systems. The non-motor symptoms are as follows:

***Neuropsychiatric*.** These symptoms, which are observed frequently in PD cases, could hurt the quality of lives of the patients as much as motor abnormalities do. The main examples of these symptoms are cognition disturbances, dementia, sleep and mood disorders. and sensory dysfunction.

Cognitive impairments can occur during early stages or even before diagnosis of PD (Caballol et al., 2007). Executive dysfunction, which can cause deficits in planning, abstract thinking, and controlling suitable and unsuitable actions, is the most common cognitive disorder and underlies other areas of this category such as language, and memory (Lees and Smith, 1983; Levin et al., 1989; Taylor and Saint-Cyr, 1995; Dubois and Pillon, 1997).

Affected patients have also been shown to have 2-6 times higher risk of dementia than the general population (Marder et al., 1995; Aarsland et al., 2001; Hobson and Meara, 2004; Caballol et al., 2007). The prevalence of dementia is almost 40% in PD cases. The main factors associated with dementia are age (Aarsland et al., 2001; Hobson and Meara, 2004), severity of motor symptoms (Marder et al., 1995; Hughes et al., 2000; Aarsland et al., 2001), and the rate of disease progression (Hughes et al., 2000; Aarsland et al., 2003; Janvin et al., 2005). Although the clinical symptoms could be similar to those of Alzheimer's disease, PD patients do not show severe aphasia (language disorder), apraxia (inability to perform learned movements although there is no physical disability), or agnosia (inability to

recognize objects, people, or smells even though the sensory system or memory is not defective) (Emre, 2003).

Sleep disorders, mostly as nighttime awakenings (Porter et al., 2008), are reported in the majority of the patients (Stacy, 2002; Gjerstad et al., 2006). The frequency of nighttime awakenings is three times higher in patients compared to the control population (Stacy, 2002). Rapid eye movement (REM) sleep behavior disorder, which can be considered as a risk factor for PD, is frequently reported (Schenck et al., 1996; Gagnon et al., 2006; Borek et al., 2007). Excessive daytime sleepiness and sleep attacks are further examples of sleep problems from which PD patients may suffer (Tandberg et al., 1999; Gjerstad et al., 2006) however, they could be, at least to some extent, due to secondary effects of the medications (Hauser et al., 2000; Ondo et al., 2001; Stacy, 2002).

Mood disturbances are more reported in these patients compared to normal cases and are usually associated with dementia (Brown and MacCarthy, 1990; Aarsland et al., 1999; Emre, 2003). The most common mood disorders are depression, anxiety, and apathy (Starkstein et al., 1992; Dell'Agnello et al., 2001; Aarsland et al., 2007).

Autonomic disturbances. Studies reveal that PD is not exclusively involved with the central nervous system. It can also be associated with a wide range of bothersome autonomic abnormalities (Chaudhuri, 2001; Hely et al., 2005; Magerkurth et al., 2005; Simuni and Sethi, 2008), whose severity increases with age and severity of the disease itself (Verbaan et al., 2007). Of those, the most prevalent are constipation, orthostatic hypotension, sweating dysfunction, bladder hyperactivity, sphincter dysfunction, and erectile dysfunction (Goldstein, 2003; Swinn et al., 2003; Allcock et al., 2004; Magerkurth et al., 2005;

Goldstein, 2006; Savica et al., 2009). Evidence suggests that autonomic problems in PD start from the periphery and may occur years before onset of motor disorders (Iwahashi et al., 1991; Abbott et al., 2001; Braak et al., 2003; Braak et al., 2004; Goldstein, 2006; Abbott et al., 2007; Gao et al., 2007; Savica et al., 2009; Goldstein et al., 2011).

Sensory disorders. The sensory system is another affected part of this complex disease. Olfactory dysfunction is the most studied and affects 70%-100% of the patients (Chaudhuri and Naidu, 2008; Simuni and Sethi, 2008). This may occur well before development of motor symptoms (Ansari and Johnson, 1975; Doty et al., 1992; Braak et al., 2004; Ponsen et al., 2004; Siderowf et al., 2007; Ross et al., 2008). Pain (mostly oral and genital; (Ford et al., 1996; Waseem and Gwinn-Hardy, 2001) and visual disorders (mostly in color perception and visual evoked potentials; (Bodis-Wollner and Yahr, 1978; Kupersmith et al., 1982; Bodis-Wollner et al., 1987; Rodnitzky, 1998; Oh et al., 2011) are other types of sensory dysfunctions described in PD.

As mentioned several times, some of non-motor symptoms precede clinical PD symptoms, making them good candidates as early markers of PD.

1.4 Pathology

1.4.1 **Pathophysiology.** Physical effects of PD in the central nervous system appear mostly as (although not limited to) death of dopaminergic neurons in the substantia nigra pars compacta (SNc) (reviewed by (Blandini et al., 2000; Obeso et al., 2008; Schapira, 2009)). The SNc is part of the midbrain and consists of neuromelanin-pigmented dopaminergic neurons, that innervate striatum in the basal ganglia. The basal ganglia's

outputs inhibit different motor areas and this constant activity inhibits initiation of movement at an inappropriate time. Specifically, motor circuits are divided into two main inhibitory pathways in the basal ganglia. One pathway, which is controlled by dopamine D1 receptors and induced by dopamine inputs from SNc, extends from the striatum and sends inhibitory inputs directly to internal globus pallidus (Gpi) (direct pathway). The second pathway, which is controlled by dopamine D2 receptors, is inhibited by SNc projections, sends its signals from the striatum to Gpi via the external globus pallidus (Gpe) and subthalamic nucleus (STN) (indirect pathway). The final outcome of the indirect pathway on Gpi is excitatory. Gpi is the output of this system and sends its inhibitory signals to thalamus. Thalamus, then, via its excitatory projections, transfers the signals to the motor cortex. Dopaminergic depletion, which occurs in PD, disrupts the balance of this system in favor of the indirect versus the direct pathways. The sum results of these events are hyperactivity of STN and inhibitory functions of Gpi, inhibition of thalamus and motor cortex at pathologically higher levels (Albin et al., 1989, 1995; Chesselet and Delfs, 1996; Parent and Cicchetti, 1998).

1.4.2 **Pathology.** As mentioned before, at the clinical level, PD presents as a motor disorder, and at the histological level it is characterized by loss of dopaminergic neurons (which contain neuromelanin) in the SNc. This is a macroscopic feature and can be observed in post-mortem tissues as a decrease of melanin pigmentation in the SNc. At the cellular and microscopic level, its hallmark is the presence of eosinophilic protein accumulations, termed Lewy bodies, in the SNc and several other areas of brain. Lewy bodies, which were first reported by Fritz Heinrich Lewy (Rodrigues e Silva et al., 2010), have an eosinophilic core with filamentous and granular structure and filamentous surroundings with radial patterns

(Roy and Wolman, 1969). These protein inclusions contain neurofilaments (Roy and Wolman, 1969; Goldman et al., 1983), ubiquitin (Kuzuhara et al., 1988; Lowe et al., 1988; Manetto et al., 1988), and alpha-synuclein (Spillantini et al., 1997; Spillantini et al., 1998) (Okazaki et al., 1961; Kosaka et al., 1976; Ince et al., 1998).

1.5 Diagnosis and treatment

PD can be diagnosed from the classical clinical manifestation, medical history, and a neurological examination. Standard criteria for definitive diagnosis have not yet been developed. Traditionally, PD is diagnosed based on the presence of two of three parkinsonian hallmarks (rest tremor, rigidity, akinesia). Furthermore, the diagnostic evaluation should exclude other neurological disorders that cause Parkinsonism. Radiological procedures such as computed tomography (CT) or magnetic resonance imaging (MRI) are useful for this purpose. Decreased uptake of striatal dopaminergic markers can be demonstrated using imaging techniques such as positron emission tomography (PET) or single-photon emission computed tomography (SPECT).

Currently there is no cure for PD. The aims of management are to ease the symptoms of the disease and to manage neurological and behavior problems. Levodopa, dopamine agonists, and inhibitors of monoamine oxidase type B (MAO-B) have been the mainstream medication therapies for PD. Other medications, such as catechol-O-methyltransferase (COMT) inhibitors, are also used. Surgical treatment mainly focuses on deep brain stimulation (DBS) of the STN, which reduces its activity and alleviates the motor symptoms of PD. For nonmotor and nondopaminergic symptoms, such as depression, selective serotonin reuptake inhibitors (SSRIs) are used. Anxiety could be managed by short-acting

benzodiazepines.

1.6 Pathogenesis

The molecular mechanism(s) underlying PD remains largely elusive. Most cases of PD (85-90%) are sporadic. Reports show that environmental factors potentially are more crucial in patients older than 50 years, and genetic factors play a more important role in younger patients (Martin et al., 1973; Richard et al., 1997; Tanner et al., 1999). Environmental factors, such as rural living, well water, and pesticide/herbicide exposure, increase the risks for PD (Barbeau et al., 1987; Semchuk et al., 1991, 1992; Gorrell et al., 1996; Seidler et al., 1996; Betarbet et al., 2000). Probably the most direct evidence for environmental role is the causative effect of 1,2,3,6-methyl-phenyl-tetrahydropyridine (MPTP) in PD (Langston et al., 1983). MPTP is a side-product of the chemical synthesis of MPPP, an illegal meperidine derivative used as a recreational drug. People that used contaminated MPPP developed PD within 7-14 days (Langston et al., 1983). However, no known environmental or naturally occurring MPTP-like causative reagent has been established for the disease.

A small percentage of cases (10-15%) are associated with known genetic factors (reviewed by (Warner and Schapira, 2003)). Although rare, they are invaluable subjects in research, as identifying their coding proteins and mechanism(s) of function enables us to reveal the biochemical pathways underlying sporadic PD. These genes and their functions will be discussed later in this chapter. ((Olanow and Tatton, 1999; Gandhi and Wood, 2005; Shadrina et al., 2010; Jannetta et al., 2011; Saxena and Caroni, 2011; Schapira, 2011; Jellinger, 2012).

Neuronal cell death in PD can be attributed to several factors such oxidative stress,

mitochondrial malfunction, excitotoxicity, neuroinflammation, and abnormal protein folding. Regardless of the potential neurotoxic mechanisms, cell death seems to take place, to some extent, via apoptotic signaling pathways. However, it remains unclear which of the above components is central to cell death/pathogenesis.

1.6.1 Oxidative stress. The toxic features of oxygen species were suggested by Gershman et al. in the theory of free radicals in 1954 (Gerschman et al., 1954). Based on this theory, the toxicity of oxygen species lies in the reduced forms of oxygen. However, it was not until 1956 when the role of free radicals in aging was suggested by Denham Harman (Harman, 1956). Since then, numerous studies have been conducted to reveal the role of free radicals in generation of oxidative stress and their effects on physiological processes. Reactive oxygen species as well as reactive nitrogen species (ROS and RNS respectively) are produced regularly in normal cellular metabolism. At physiological levels they can have beneficial roles, such as immune responses against pathogens and induction or inhibition of several cell signaling pathways (Lowenstein et al., 1994; Taylor-Robinson et al., 1994; Bae et al., 1997; Neufeld et al., 1999; Chandel et al., 2000; Catarzi et al., 2002; Arana et al., 2012; Sinenko et al., 2012). Excessive amounts, though, could be hazardous to cells.

The net hazardous effect of free radicals, which leads to damage to cells and tissues, is termed oxidative stress. These stresses are caused by overproduction of free radicals or malfunction of enzymatic or non-enzymatic cellular antioxidant systems.

1.6.1.1 Reactive oxygen species and their production. By definition, free radicals are molecules which have one or more unpaired electron(s) in their atomic or molecular orbital. This unpaired electron(s) possesses a significant level of reactivity. The free radicals

generated from oxygen are termed reactive oxygen species (ROS) and are the most important group of radicals in biological systems (Miller et al., 1990). The primary ROS, which is generated by addition of one electron to the molecular oxygen, is superoxide ($O_2^{\cdot -}$) (Miller et al., 1990). Superoxide has the ability to interact with other molecules and create secondary ROS, either directly or through catalyzed processes (which can be enzymatic or metal-mediated) (reviewed by(Valko et al., 2005)) (Figure 1.1).

The site of superoxide formation in the cells is mainly the mitochondrial electron transport chain (ETC) (Cadenas and Sies, 1998). ETC is the major generator of ATP in mammalian cells. In the process of electron transportation in ETC, a slight proportion (up to 1-3%) of electrons leaks from the system to interact with oxygen and yield superoxide. Within the ETC, superoxide production occurs in Complexes I and III. Superoxide generated by Complex I, unlike that of Complex III, is released into the mitochondrial matrix and does not penetrate to cytosol (Muller et al., 2004). NADPH oxidase and xanthine oxidase are other sources of superoxide formation.

One of the examples of secondary ROS is hydroxyl radical, $^{\cdot}OH$, which is well known for its high reactivity and short half-life (Pastor et al., 2000). Superoxide is involved in production of hydroxyl radicals via multiple pathways. Following stress conditions in the cells, excess superoxide can induce release of iron from molecules harboring iron, such as dehydratases with [4Fe–4S] clusters (Liochev and Fridovich, 1994). The released Fe^{2+} mediates generation of hydroxyl radicals from hydrogen peroxide in a reaction termed the Fenton reaction (Leonard et al., 2004; Valko et al., 2005). Superoxide can induce the hydroxyl radical generation in a second mechanism, by reducing Fe^{3+} to Fe^{2+}, which catalyzes the Fenton reaction. Superoxide also takes part in the Haber–Weiss reaction, where it directly

interacts with hydrogen peroxide and generates hydroxyl radicals (Kehrer, 2000).

Fenton reaction

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$$

Providing Fe^{2+}, the catalyzer of Fenton reaction, by superoxide

$$Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2$$

Haber-Weiss reaction

$$O_2^{\bullet -} + H_2O_2 \rightarrow O_2 + OH^{\bullet} + OH$$

The other secondary ROS produced in biologic systems are peroxyl radicals (ROO·), particularly the hydroperoxyl radical, also known as the perhydroxyl radical (HOO·). This is the protonated superoxide which represents a small portion (0.3%) of total cytosolic superoxide (De Grey, 2002).

1.6.1.2 Oxidative damage to cellular components. The cellular structures that could be targets of oxidative damage are nucleic acids, lipids and proteins (Valko et al., 2006). All DNA/RNA sub-structures, including the sugar backbone and purine and pyrimidine bases, could be attacked by hydroxyl radicals. The best studied product of oxidative-DNA modification is 8-hydroxyguanosine (8-OH-G), which could be used as a marker of oxidative stress (Nunomura et al., 1999) (Figure 1.1).

Oxidative damage in lipids mostly involves peroxidation of polyunsaturated fatty acids in phospholipids (Siems et al., 1995). The produced peroxyl radicals (ROO·) may undergo cyclisation and reform to endoperoxides and eventually to malondialdehyde (MDA). MDA

is one of the final products of lipid peroxidation and acts as an internal mutagen in cells by interacting with adenine, guanine and cytosine (Wang et al., 1996; Fink et al., 1997; Mao et al., 1999). 4-hydroxy-2-nonenal (HNE) is another product of lipid peroxidation that is known to be the main toxic yield of lipid peroxidation (Figure 1.1).

The backbone of proteins and side chain of any amino acid, particularly cysteine and methionine, could be subjected to oxidative damage (Garrison et al., 1962; Schuessler and Schilling, 1984; Huggins et al., 1993; Neuzil et al., 1993). The result of oxidative damage to the proteins' backbone is cleavage of peptide bond and on different amino acid side chains is formation of different products:

Phenylalanine → Mono- and dihydroxy by-products (Maskos et al., 1992; Huggins et al., 1993; Kaur and Halliwell, 1994).

Tyrosine → 3,4-dihydroxyphenylanine, nitrotyrosine, chlorotyrosine and tyrosyl radicals (Fletcher and Okada, 1961; Beckman et al., 1992; Maskos et al., 1992; Simpson et al., 1993; Wells-Knecht et al., 1993), (Giulivi and Davies, 1993; Heinecke et al., 1993; Domigan et al., 1995; Eiserich et al., 1996).

Tryptophan → *N*-formyl-kynurenine, 3-hydroxy-kynurenine, oxindole, and hydro-pyrroloindole (Winchester and Lynn, 1970; Kuroda et al., 1975; Guptasarma et al., 1992).

Histidine → 2-oxohistidine and 4-OH-glutamate (Farber and Levine, 1986; Uchida and Kawakishi, 1993; Berlett et al., 1996).

Lysine, proline, arginine, and threonine residues → carbonyl by-products for each case (Amici et al., 1989).

Cysteine and methionine → disulphide cross-links between proteins' thiol groups ($P^1S–SP^2$).

Cysteine and methionine are sulfur-containing residues and of the examples above, they are especially prone to oxidation (Mudd et al., 1969; Takahashi and Goto, 1990; Zhou and Gafni, 1991; Mohr et al., 1994). Their oxidation is, however, reversed by glutathione (GSH).

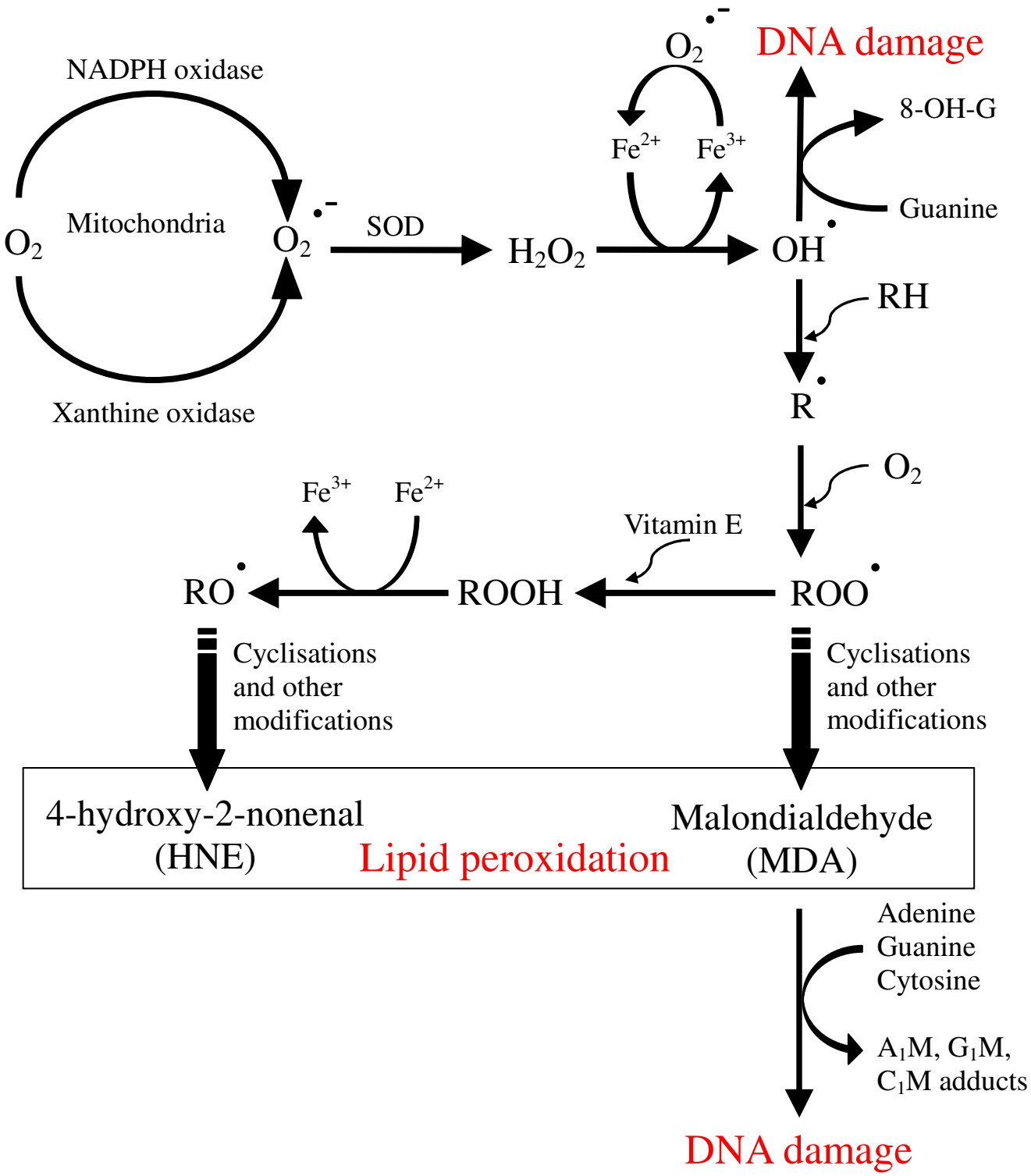

NADPH oxidase
Mitochondria
Xanthine oxidase
O_2
$O_2^{\bullet -}$
SOD
H_2O_2
$O_2^{\bullet -}$
Fe^{2+}
Fe^{3+}
DNA damage
8-OH-G
Guanine
OH•
RH
R•
O_2
ROO•
Vitamin E
ROOH
Fe^{3+}
Fe^{2+}
RO•
Cyclisations and other modifications
Cyclisations and other modifications
4-hydroxy-2-nonenal (HNE)
Lipid peroxidation
Malondialdehyde (MDA)
Adenine
Guanine
Cytosine
A_1M, G_1M, C_1M adducts
DNA damage

Figure 1.1

Figure 1.1: ROS production and oxidative damage to cellular components. Superoxide is generated through reduction of molecular oxygen by NADPH oxidases, xanthine oxidase and mitochondrial ETC. Superoxide dismutase (SOD) converts superoxide to hydrogen peroxide, which will be broken down to hydroxyl radical, $OH^{\bullet}$, by Fe^{2+} or Cu^{+}, through Fenton reaction. $OH^{\bullet}$ is very reactive and could cause DNA damage. It can also interact with polyunsaturated fatty acids, RH, and reduce them to carbon-centred lipid radicals, $R^{\bullet}$. $R^{\bullet}$ interacts with oxygen and generates lipid peroxyl radical, $ROO^{\bullet}$, which can go through lipid peroxidation reactions. Following this process, the lipid peroxyl radical could go through several cyclisation and reduction reactions and generates malondialdehyde, MDA, or be reduced to lipid hydroperoxide, ROOH, by vitamin E, then to lipid alkoxyl radicals, $RO^{\bullet}$, by Fe^{2+}. Lipid alkoxyl radical will undergo several cyclisation and other modification processes to generate lipid peroxides, for example 4-hydroxy-nonenal, when arachidonic acid is the substrate of peroxidation. MDA can eventually cause DNA damage by interacting with Cytosine, Adenine, and Guanine and producing adducts M1C, M1A and M1G.

1.6.1.3 The role of oxidative stress in PD pathogenesis. As mentioned before, oxidative stress has a high potential to damage cellular structures and eventually disrupt their physiological functions. For instance, oxidative alteration of proteins may result in disruption of enzyme activities including the proteolytic function of proteases that digest oxidized proteins (Rivett, 1986; Oliver et al., 1987; Zhou and Gafni, 1991; Szweda and Stadtman, 1992; Dean et al., 1993; Giulivi and Davies, 1993). Oxidative stress also causes several protein-protein cross-links such as -S-S- and -Tyr-Tyr- between sulfur-containing or tyrosine-containing proteins, respectively.

This destructive potential makes oxidative stress an important subject of study in ageing process and several diseases including PD. Indeed, there is a respectable bulk of evidence from *in vitro*, *in vivo,* and human studies that supports the involvement of oxidative stress in PD. This evidence is as follows:

A) Dopamine increases the risk of oxidative stress. Non-enzymatic autoxidation and monoamine oxidase (MAO) metabolism of dopamine generates hydrogen peroxide (H_2O_2) and other ROS (Olanow, 1990, 1993). As the SNc is one of the major producers of dopamine neuro-transmitter and the progressive death of dopaminergic neurons is associated with elevation of dopamine production and turnover in surviving neurons (Fornstedt et al., 1990), oxidative stress is believed to be prevalent in these neurons.

Enzymatic oxidation of dopamine

$$\text{Dopamine} + O_2 + H_2O \ (\text{MAO}) \rightarrow \text{3,4 dihydroxyphenylacetaldehyde}$$

$$+ NH_3 + H_2O_2$$

Auto-oxidation of dopamine (SQ, semiquinone)

$$\text{Dopamine} + O_2 \rightarrow SQ^{\cdot} + O_2^{-} + H^{+}$$

$$\text{Dopamine} + O_2^{-} + 2H^{+} \rightarrow SQ^{\cdot} + H_2O_2$$

B) Oxidative damage is increased in PD tissues. The SNc of PD patients demonstrate evidence of oxidative stress. This includes the elevation of oxidative damage in lipids (lipid hydroperoxides), proteins (protein carbonyls), and DNA (8-hydroxy guanine) (Dexter et al., 1994; Alam et al., 1997a; Alam et al., 1997b). In addition, heightened free iron and decreased ferritin, the non-reactive iron reservoir, are observed in the substantia nigra (SN) of PD patients (Dexter et al., 1991). Increased reactive iron activates the Fenton reaction and produces ROS. This is particularly important in dopaminergic neurons, as one of the side-products of dopamine autoxidation, neuromelanin (Graham, 1978, 1979), accumulates iron (Enochs et al., 1994).

C) Antioxidant defense is lower in PD tissues. The activities of glutathione peroxidase and catalase and levels of reduced glutathione appear to decrease in PD SN (Ambani et al., 1975; Hornykiewicz and Kish, 1987; Sian et al., 1994). This suggests that the antioxidant defense in these cases, at least to some extents, is impaired.

D) Parkinsonism-causing toxins are oxidants. Neurotoxins that exclusively target and degenerate dopaminergic neurons in the SNc, such as MPTP, or toxins which are associated with the increased risk of PD, such as rotenone, exert their toxicity though promoting oxidative stress (Langston et al., 1983; Nicklas et al., 1985; Betarbet et al., 2000).

1.6.2 Mitochondrial dysfunction. Mitochondria are vital micro-organelles that possess unique structural and functional characteristics. Their most important role is energy production by oxidative phosphorylation. Besides that, they have a central role in the maintenance of calcium homeostasis, execution of several biochemical pathways such as pyruvate oxidation and the citric acid cycle, production of amino acids and fatty acids, and regulation of programmed cell death pathways. Thus, it is expected that mitochondrial dysfunctions lead to cellular impairments and eventually degeneration.

1.6.2.1 The evidence for mitochondrial malfunction in PD pathogenesis.

A) Parkinsonism-causing toxins disrupt mitochondrial function. The first line of evidence suggesting the role of mitochondrial dysfunction in PD pathogenesis was introduced in the late 1970s and early 1980s, when Davis *et.al* and Langston *et.al* reported MPTP, the previously mentioned side-product of MPPP production and an inhibitor of complex I of the electron transport chain (ETC) , caused parkinsonian symptoms in drug addicts abusing MPPP (Davis et al., 1979; Langston et al., 1983). The mechanisms underlying the toxic effects of MPTP following impairment of electron transfer involves loss of ATP synthesis, increased production of ROS and RNS (Hasegawa et al., 1990; Chan et al., 1991; Hantraye et al., 1996; Fabre et al., 1999; Pennathur et al., 1999; Drechsel and Patel, 2008), activation of apoptotic cascades including pro-apoptotic members of Bcl-2 family, JNK, and caspases (Hartmann et al., 2000; Hartmann et al., 2001; Perier et al., 2005; Perier et al., 2007). Similar to sporadic PD cases, MPTP-related patients had dopaminergic loss in the SNc but no sign of Lewy bodies in that area (Davis et al., 1979; Langston et al., 1999). Importantly, in both of these groups of patients, relatively similar positive and counter effects of L-DOPA therapy is observed.

In addition, epidemiological studies demonstrate that pesticides and environmental toxins such as rotenone and paraquat increase the risk of PD (Gorell et al., 1998). These toxins have been shown to be inhibitors of complex I and to cause dopaminergic loss in rodent models of PD (Betarbet et al., 2000; Thiruchelvam et al., 2000).

B) Mitochondrial dysfunction is observed in PD tissues: Studying platelets of PD patients (Parker et al., 1989; Krige et al., 1992; Haas et al., 1995) and post mortem tissues obtained from the SNc (Schapira et al., 1989; Schapira et al., 1990; Janetzky et al., 1994) and frontal cortex (Keeney et al., 2006; Parker et al., 2008) of PD patients reveals that complex I activity is reduced. These studies further support the critical role of mitochondrial impairment in PD pathogenesis.

C) Mitochondrial genome mutations. One of the unique features of the mitochondrion is its double-stranded circular genome and replication cycles independent of nuclear replication. Thirteen proteins, all of which are components of ETC, are encoded by mitochondrial DNA (mtDNA) (Reeve et al., 2008). This shows the importance of normal structure and function of this genome. Interestingly, there are higher levels of mtDNA mutations in the SNc of aged people and PD patients compared to the control groups (Bender et al., 2006; Kraytsberg et al., 2006). This seems to occur in a specific manner, as such an accumulation was not observed in other brain areas, including the hippocampus, cerebral cortex, and cerebellum, of these individuals. Another piece of evidence supporting the link between mtDNA mutations and dopaminergic loss is an *in vitro* study, in which the mitochondrial transcription factor A (TFAM) was conditionally knocked out in dopaminergic neurons of mouse midbrain. In these mice, respiratory chain deficiency, neuronal loss and L-dopa-responsive motor dysfunctions were observed (Ekstrand et al., 2007).

D) PD genes and mitochondria. Inherited forms of PD include up to 10% of all PD cases. Our knowledge about the biochemical roles of PD genes supports the hypotheses proposed for the molecular mechanisms involved in sporadic forms of PD. Indeed, a number of these genes play critical roles in mitochondrial integrity, response to stresses and quality control (Cookson, 2005; Abeliovich and Flint Beal, 2006; Wood-Kaczmar et al., 2006; Burke, 2008; Fitzgerald and Plun-Favreau, 2008). For instance, PINK1 deficiency disrupts mitochondrial trafficking, membrane potential, calcium influx, reduces complexes I and IV activities and ATP production, elevates ROS generation, and causes abnormalities in mitochondrial morphology (Gautier et al., 2008; Wood-Kaczmar et al., 2008; Gegg et al., 2009; Gispert et al., 2009; Grunewald et al., 2009; Liu et al., 2009b; Marongiu et al., 2009; Morais et al., 2009; Weihofen et al., 2009). Pathogenic mutations in the Parkin gene decrease complex I activity (Muftuoglu et al., 2004) and increase vulnerability to certain mitochondrial toxins (Darios et al., 2003; Hyun et al., 2005; Casarejos et al., 2006; Rosen et al., 2006; Vercammen et al., 2006). Parkin also protects mitochondria from swelling and cytochrome *c* release, induces mtDNA repair, and protects mtDNA against oxidative damage (Darios et al., 2003; Berger et al., 2009; Rothfuss et al., 2009). DJ-1 deficient cells show impaired mitochondrial dynamics (Irrcher et al., 2010) and reduced mitochondrial ROS scavenging (Andres-Mateos et al., 2007). Mitochondrial localization of wild type or pathogenic mutants of α-synuclein reduces complex I activity and increases ROS production (Devi et al., 2008), cytochrome **c** release, and mitochondrial calcium influx (Parihar et al., 2008). α-synuclein also inhibits complex IV activity, impairs membrane potential and increases the oxidative damage of mitochondrial metabolic proteins (Hsu et al., 2000; Song et al., 2004; Poon et al., 2005).

The functions and the biochemical roles of these genes will be discussed on detail in the next

sections of this dissertation.

1.6.3 Excitotoxicity. Excitotoxicity is induced by excess amount of excitatory neurotransmitters. The main neurotransmitter associated with excitotoxicity is glutamate. Glutamate is the most abundant excitatory neurotransmitter in the central nervous system, where it acts its role through several receptors including N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazole proprionic acid (AMPA) , mGlu1, and mGlu8 (Greenamyre and Porter, 1994; Dingledine et al., 1999). The excitotoxicity pathways are mostly induced by activation of NMDA or AMPA receptors, followed by stimulation of voltage gated calcium channels and influx of calcium from extra-cellular reservoirs, which eventually triggers calcium release from endoplasmic reticulum. High levels of glutamate in the synaptic cleft can trigger these events and lead to neuronal death mostly through disruption of calcium homeostasis. This pathogenically elevated concentration of calcium causes over-production of nitric oxide radicals and ROS (Coyle and Puttfarcken, 1993; Dugan et al., 1995), mitochondrial dysfunction (Gunter et al., 1994; Dugan et al., 1995) and activation of proteases (Nicotera and Orrenius, 1992; Orrenius et al., 1992). Excitotoxicity in PD and other chronic neurodegenerative diseases could have more complicated routes (Albin and Greenamyre, 1992; Greene and Greenamyre, 1996). A possible mechanism could be that disruption of neuronal membrane polarity removes the $Mg2^{+}$-mediated blockade of NMDA receptors in a voltage dependent manner (Zeevalk and Nicklas, 1992). In turn, this leads to activation of NMDA receptors, extra-cellular calcium influx, and finally induction of excitotoxicity-type neuronal death. Disruption of neuronal membrane polarity may occur by inhibition of Na^{+},K^{+}-ATPase due to, for instance, mitochondrial defects. Indeed, there are several reports which show that the NMDA receptor mediates the toxicity induced by

Na^+,K^+-ATPase inhibition (Abele and Miller, 1990; Lees et al., 1990; Brines and Robbins, 1992).

The importance of glutamate-mediated excitotoxicity in PD increases due to the notion that glutamate is the main excitatory neuro-transmitter in basal ganglia (Greenamyre and Porter, 1994) and one of the targets of STN glutamatergic projections is the SNc (Rodriguez et al., 1998). The series of events in PD start with SNc neuronal loss, followed by reduction of dopaminergic messages from the SNc to the striatum and hyperactivity of the STN. The end result is an increase in glutamate release to the SNc dopaminergic neurons (Rodriguez et al., 1998). This could be one of the processes leading to progression of the disease.

1.6.3.1 The evidence of involvement of excitotoxicity in PD.

A) Elevation of glutamate in rodent models of PD. The interruption of glutamate homeostasis in the chronic mouse model of PD provides evidence of the role of glutamate and excitotoxicity in PD. In this regard, it has been reported that the extracellular concentration of glutamate increases in the SN of mice that are chronically exposed to MPTP (Meredith et al., 2009).

B) Inhibition of excitatory receptors reduces PD symptoms. Experimental inhibition of excitotoxic pathways provides evidence that these pathways play a role in PD pathogenesis. Remacemide hydrochloride and NBQX, antagonists of NMDA and AMPA receptors, ameliorate PD symptoms in rodent and monkey models of PD (Klockgether et al., 1991; Greenamyre et al., 1994). MGlu5 receptor antagonists also improve the motor symptoms in rat and mouse models of PD (Ossowska et al., 2001; Breysse et al., 2002).

C) Inhibition of excitotoxicity protects dopeminergic neurons. A number of studies have shown that inhibition of excitotoxicity, besides improving PD symptoms, protects neurons against *in vivo* models of PD. As mentioned before, the SNc is one of the targets of STN glutamatergic fibers, and hyperactivity of STN activity (what occurs in PD) is associated with increase of excitotoxicity in the SNc. Reduction of STN function, by lesioning or deep brain stimulation (DBS), protects dopaminergic neurons of the SNc up to 24% in monkeys treated with MPTP (Wallace et al., 2007). In addition, agonists of MGlu2, MGlu3 and MGlu5 receptors also have protective roles against MPTP toxicity in rat and mice (Battaglia et al., 2003; Battaglia et al., 2004; Corti et al., 2007).

1.6.4 Neuroinflammation. For a long time it was thought that brain was incapable of an inflammatory response because of the existence of the blood brain barrier and absence of any lymphatic system. Presently, however, it is known that stimulants such as pathogens, trauma, and stroke can trigger immune responses in the CNS. Microglia are a type of glial cell responsible for innate responses (Wersinger and Sidhu, 2002; Wyss-Coray and Mucke, 2002). Activation of microglia at moderate levels has beneficial results, such as removing neurotoxins, mediating axonal growth by clearing unhealthy or dead cells, and producing trophic factors such as BDNF (Aloisi, 1999; Batchelor et al., 1999; Nakamura, 2002; Orr et al., 2002). In response to a longer exposure to stimuli, microglia generate ROS, RNS, and cytokines. Extended presence of the stimulus leads to microglia becoming anchored to neurons (Kreutzberg, 1996) and finally exerting their scavenging role (Beyer et al., 2000). At this step, major histocompatibility complex (MHC), CD40, and CD80 are also up-regulated which induces even more powerful inflammatory responses (Aloisi et al., 2000). The potential triggers of microglia activation in the CNS are lipopolysaccharides (LPS), toxins

(de Meira Santos Lima et al., 2006), accumulated cellular components which are aberrantly modified, and molecules produced by vulnerable dopaminergic neurons (Le et al., 2001).

As mentioned before, exertion of oxidative damage is one of the consequences of microglial activation. This could be through production and release of ROS by nicotinamide adenine dinucleotide phosphate oxidase (NDPH oxidase) or modulation of arachidonic acid signaling by cyclooxygenase (COX) and lipoxygenase (LOX). NADPH oxidase, an enzyme that produces superoxide (O_2^-) from molecular oxygen, is the main source of ROS production in activated microglia, and increased superoxide concentration has toxic effects against neurons (Gao et al., 2003a; Wu et al., 2005). COX facilitates production of prostaglandins from arachidonic acids. COX-1 is upregulated upon inflammation and is the main cyclo-oxygenase in microglia (Ju and Neufeld, 2002; Schwab et al., 2002). COX-2 is involved in several cellular pathways such as oxidation of cytosolic dopamine (Hastings, 1995), and its upregulation is induced in neurons and astrocytes by CNS injuries (Consilvio et al., 2004). One of the products of COX-2 is prostaglandin E2. which can act as a neurotoxin in dopaminergic neurons (Gao et al., 2003a). Some prostaglandins cause oxidative stress by inhibition of glutathione and glutathione peroxidase and reduction of mitochondrial membrane potential. Besides oxidative stress, inflammation could mediate neuronal loss through phagocytosis by microglia. This could be triggered by neuron-produced chemokines such as MCP-1 (Aloisi et al., 2000; Aloisi, 2001) and accumulation or release of misfolded or oxidized proteins, such as **α**-synuclein (Zhang et al., 2005b), in neurons. Another inducer of phagocytosis, which is especially important in PD pathogenesis, is release of neuromelanin, a side product of dopamine synthesis, from damaged dopaminergic neurons (Wilms et al., 2003; Zucca et al., 2004; Kim and Joh, 2006; Zecca et al., 2006).

In summary, short-term inflammation is mostly associated with production of neuroprotective factors by microglia and leads to tissue repair (Wyss-Coray and Mucke, 2002) (Marchetti and Abbracchio, 2005). While, extended neuroinflammation elevates vulnerability and adverse results.

Some features of the midbrain, such as relatively high levels of microglia (Lawson et al., 1990) and high levels of pro-inflammatory cytokines, oxidative stress, and misfolded proteins make the dopaminergic neurons of the SNc prone to these inflammatory events.

1.6.4.1 The evidence of involvement of neuroinflammation in PD pathogenesis.

A) Inflammation in PD patients. Evidence collected since late 1980's supports the contribution of inflammation to dopaminergic loss in patients with sporadic PD. The first evidence, demonstrated by McGeer *et al,* was the upregulation of MHC and elevation of activated microglia population in PD cases (McGeer et al., 1988).

The elevation of pro-inflammatory factors or other molecular markers of inflammation was then reported in PD patients. These factors include presence of MHC light-chain (**β**2-microglobulin) in the striatum of patients (Mogi et al., 1995); antibodies against proteins that were modified by the products of dopamine oxidation (Rowe et al., 1998); proinflammatory cytokines including tumor necrosis factor-**α** (TNF-**α**), interleukin-1**β** and 6 (IL-1**β,** IL-6) in the cerebrospinal fluid (CSF) and striatum of PD brains (Mogi et al., 1994; Blum-Degen et al., 1995; Muller et al., 1998); TNF-**α**, IL-1**β,** COX-2 and inducible nitric oxide synthase (iNOS) in activated glia of the SNc of PD cases (Hunot et al., 1996; Hirsch et al., 1998; Knott et al., 2000); and ICAM-1 in activated astrocytes of the SNc of patients, especially around neuronal debris in spots with massive neuronal loss (Miklossy et al.,

2006). Interestingly, activated microglia were also observed in post-mortem tissue obtained from the brain of an MPTP-induced PD patient 16 years after disease onset (Langston et al., 1999), suggesting that the inflammatory response in PD could be ongoing and a factor in the progression of the disorder.

B) Inflammation in animal models of PD. Activated microglia are observed in several animal models of PD, such as MPTP, rotenone, and 6-hydroxydopamine (6-OHDA) (Czlonkowska et al., 1996; Cicchetti et al., 2002; Gao et al., 2002b). In addition, NADPH oxidase and iNOS expression are both increased in these models (Liberatore et al., 1999; Iravani et al., 2002; Wu et al., 2003), whose inhibition (Hemmer et al., 2001; Wu et al., 2003; Kim and Joh, 2006), and also inhibition of COX-2 (Feng et al., 2002) reduces neuronal death.

Interestingly, while LPS results in activation of microglia and dopaminergic loss in the rat SNc (Castano et al., 1998; Herrera et al., 2000; Gayle et al., 2002; Gao et al., 2003b), it does not show toxicity in other regions such as the cortex and hippocampus (Kim et al., 2000), or in other types of neurons such as GABAergic and serotonergic neurons (Herrera et al., 2000; Gao et al., 2002a).

There are several pieces of evidence indicating the toxic function of pro-inflammatory factors, including TNF-**α**, IL-1 and IL-6 in neurons (Fisher et al., 2001; Gayle et al., 2002; Sriram et al., 2002). Supportive of this evidence are studies which show that the inhibition of the genes playing a role in performance of these factors such as TNF-**α** receptors could reduce the toxicity of MPTP in dopaminergic neurons of mice (Sriram et al., 2002).

Finally, α-synuclein, a component of Lewy bodies, has been shown to activate microglia,

induce production of extracellular superoxide, and increase morphological alterations and phagocytic function of microglia (Zhang et al., 2005b). Thus, release of this molecule from dying neurons triggers a powerful microglial response (Block and Hong, 2005).

In short, numerous lines of evidence indicate that inflammation plays an important role in initiation and/or progression of dopaminergic loss in PD.

1.6.5 Unfolded proteins and abnormal proteolysis. The pathological events impairing normal protein folding in the endoplasmic reticulum (ER) lead to ER stress. In responses to this stress a series of pro-survival pathways are activated, which are collectively termed unfolded protein response (UPR). Following UPR activation, cell protein synthesis ceases and the mechanisms responsible for handling the aggregation of unfolded proteins are triggered. These handling mechanisms include upregulation of ER chaperones and protein degradation pathways. Like other cellular stresses, if extended, ER stress will lead to toxicity, apoptosis and finally cell death.

To detect and defend against ER stress, three main systems have been developed in mammalian cells: IRE1, PERK and ATF6. These proteins are activated upon aggregation of unfolded proteins in the ER. IRE1 is a kinase with RNAse activity. In response to ER stress it is autophosphorylated and its RNAse activity is triggered, which leads to splicing and activating of a transcription factor (XBP1) and its anti-apoptotic target genes (Calfon et al., 2002; Hetz and Glimcher, 2009; Gupta et al., 2010). IRE1 can also protect against ER stress by induction of autophagy through activation of JUN N-terminal kinase (JNK)(Urano et al., 2000; Ogata et al., 2006). PERK is a kinase whose activated form reduces translation through phosphorylation of the α subunit of eukaryotic translation initiation factor 2 α

(eIF2α) (Harding et al., 1999). It also activates NRF2 (Cullinan and Diehl, 2006), an antioxidant transcription activator, and induces expression of several detoxifying enzymes (Cullinan and Diehl, 2006). ATF6 is a transcription factor (Haze et al., 1999; Schroder and Kaufman, 2005) and exerts its defensive function in ER stress conditions through activation of target genes that include chaperones and folding enzymes (Wu et al., 2007a) .

Like other cellular stresses, if extended, ER stress leads to apoptosis and cell death. This process is believed to be through deregulation of mitochondrial membrane potential, formation of permeability transmembrane pores (PTP), release of cytochrome c, and activation of pro-caspase-9 and its downstream caspases such as caspase-3, caspase-7 and caspase-6 (Olson and Kornbluth, 2001; Bao and Shi, 2007; Szegezdi et al., 2009). Caspase-2 is also involved in apoptosis triggered by excessive ER stress (Upton et al., 2008).

1.6.5.1 The evidence of involvement of UPR dysfunction in PD.

A) Protein aggregations in PD patients. The primary result of UPR dysfunction is aggregation of unfolded proteins. This pathologic accumulation has been reported in many neurodegenerative disorders (Gorman, 2008; Soto and Estrada, 2008; Winklhofer et al., 2008). In PD, Lewy bodies are the most important example of these aggregations and the main histological hallmarks of the disease (Roy and Wolman, 1969; Goldman et al., 1983; Kuzuhara et al., 1988; Spillantini et al., 1997; Brundin et al., 2008; Gorman, 2008; Rodrigues e Silva et al., 2010). In addition, post mortem studies also revealed the induction of ER stress and UPS in PD. To be more specific, the markers of these events, such as phosphorylated PERK and phosphorylated eIF2α, increase in the SNc of PD patients (Hoozemans et al., 2007).

B) UPR dysfunction and PD genes. The second line of evidence involves the PD genes, primarily α-synuclein. Mutations in the α-synuclein gene result in dominant familial PD. In the process of α-synuclein misfolding, its tertiary structure changes from mostly α-helix to β-sheet. Studies have shown that the A53T mutation in this protein increases the markers of UPR, such as CHOP, GRP78 and phosphorylated eIF2α (Smith et al., 2005a). This UPR, however, is pro-death and switches the cell's fate towards apoptosis, as blockade of phosphorylation of eIF2α protects neurons against A53T α-synuclein (Smith et al., 2005a). Mutations in LRRK2 appear to disrupt pathways involved in protein degradation ((Tong et al., 2010; Tong et al., 2012). The result of this disruption is accumulation of α-synuclein, ubiquitinated proteins, and oxidized proteins (Tong et al., 2010). Mutations in UCH-L1, also a PD gene, block degradation of α-synuclein through chaperone-mediated autophagy (CMA) and cause accumulation of this protein (Kabuta et al., 2008). Parkin is an E3 ligase, which ubiquitinates its target proteins, leading them to proteasomal degradation (Shimura et al., 2000). Loss of function mutations in Parkin result in accumulation of target proteins (Imai et al., 2000). This could be the pathogenic pathway that leads individuals with Parkin mutations to PD. Interestingly, Parkin expression has been shown to increase in a compensatory manner to manage ER stress (Bouman et al., 2011).

C) Parkinsonism-causing toxins and UPR. The third line of evidence includes reagents causing dopaminergic loss. It has been shown that Salsolinol, 1-benzyl-1,2,3,4-tetrahydroisoquinoline (1BnTIQ), 6-hydroxydopamine (6-OHDA), and 1-methyl-4-phenylpyridinium (MPP^+) up-regulate different markers of ER stress and UPR, such as ER chaperones, phosphorylated PERK, and phosphorylated eIF2 alpha, in dopaminergic cell lines (Holtz and O'Malley, 2003; Kheradpezhouh et al., 2003; Yamamuro et al., 2006).

Paraquat, an agricultural herbicide that increases the risk of PD (Sanchez-Ramos et al., 1987; Hertzman et al., 1990; Liou et al., 1997), also activates ER stress and the IRE1 pathway in human dopaminergic cell lines (Yang et al., 2009).

1.7 PD genes

During the last two decades, technological innovations in molecular biology and genetics resulted in identification, mapping, and cloning of a handful of genes whose mutations cause familial diseases. These findings include causative genes of familial PD, which are autosomal recessive or autosomal dominant. Studying of these genes has developed new attitudes towards the etiology of PD, as it has revealed the mechanisms and molecular pathways involved in the disease.

The genes linked to the major forms of familial PD and their molecular roles in the context of mitochondrial function, antioxidant defense, and protein turnover will be briefly reviewed.

1.7.1 Autosomal dominant PD.

1.7.1.1 PARK1 (α-Synuclein) (SNCA). SNCA was identified as the first PD gene by Polymeropoulos, *et al*., in an Italian family with an autosomal dominant form of inherited PD (Golbe et al., 1990; Polymeropoulos et al., 1996; Polymeropoulos et al., 1997). The protein encoded by SNCA gene, αSYN, is a cytosolic and membrane-bound protein (McLean et al., 2000) with 140 amino acids and 20 kDa molecular weight. It forms homo-tetramers (Bartels et al., 2011) and is localized in pre-synaptic terminals (Iwai et al., 1995). αSYN is also the main ingredient of Lewy bodies, the hallmark of familial and sporadic PD.

Its primary structure contains three regions: 1- the N-terminus is composed of amphipathic α-helices and has the potential for interacting with lipid structures (Fortin et al., 2004); 2- the amyloidogenic NAC region is composed of 34 residues, which are hydrophobic and responsible for protein aggregation; and 3- the C-terminus, which contains acidic and proline residues and is believed to be involved in protein-protein interactions. The pathogenic forms of αSYN are rich in β-sheet structures, more stable, and capable of generating oligomer accumulations (Goedert et al., 1998; Karpinar et al., 2009).

The causative point mutations in SNCA are A53T (Polymeropoulos et al., 1997) and A30P (Kruger et al., 1998). While these mutations are in coding regions (Berg et al., 2005b), mutations in non-coding regions have been reported in sporadic PD (Mueller et al., 2005; Mizuta et al., 2006). In addition, duplication (Ibanez et al., 2009) and triplication (Singleton et al., 2003) of SNCA leads to autosomal dominant PD due to an increase in the levels of αSYN, 1.5 and 2 times respectively (Farrer et al., 2004). This suggests that the pathogenic function of αSYN is not limited to its structurally abnormal forms. In this regard it has been shown that αSYN over-expression causes PD in a dose dependent manner, where triplicate SNCA cases demonstrate more severe and earlier onset PD compared to duplicate SNCA cases (Fuchs et al., 2007).

Transgenic mice over-expressing wild type αSYN develop neuronal loss and Lewy body-like inclusions (Masliah et al., 2000). Mice expressing A53T mutations demonstrate severe motor disorder in addition to neuronal loss and Lewy body-like aggregation (Giasson et al., 2002). This is not, however, observed in A30P transgenic mice. In line with the gain of function hypothesis, knocking out SNCA does not lead to dopaminergic loss (Abeliovich et al., 2000; Thomas et al., 2011). Transgenic models of SNCA are not restricted to mice.

Expression of human wild type or mutant αSYN in Drosophila leads to dopaminergic loss, motor dysfunction and filamentous inclusions in neurons (Feany and Bender, 2000). In spite of important information obtained from transgenic models, the physiological function of αSYN is yet to be fully uncovered.

Under physiological conditions, αSYN appears to have roles in release of neurotransmitter-containing vesicles (Murphy et al., 2000; Burre et al., 2010) and maintaining the function and morphology of the membranous organelles. αSYN over-expression leads to Golgi fragmentation (Fujita et al., 2006), lysosome swelling (Meredith et al., 2002), and ER stress (Cooper et al., 2006; Colla et al., 2012). These findings, along with the presence of organelle-derived lipids in Lewy bodies (Gai et al., 2000; Auluck et al., 2010), suggest that binding of αSYN oligomers to the membranes disrupts their structure and stability (Madine et al., 2006; Varkey et al., 2010; Giehm et al., 2011).

Another group of studies suggests that the mechanism of αSYN toxicity is through inhibition of the proteasome pathway by the aggregates (Stefanis et al., 2001; Tanaka et al., 2001; Snyder et al., 2003; Brown, 2010). Evidence that support this hypothesis include: impairment of proteasome by wild type or mutant αSYN (Stefanis et al., 2001; Snyder et al., 2003), involvement of Parkin, another PD gene, in ubiquitination and degradation of αSYN, Parkin's capability to reverse αSYN toxicity (Petrucelli et al., 2002; Oluwatosin-Chigbu et al., 2003), and co-localization of ubiquitin and αSYN in Lewy bodies (Gai et al., 2000; Sharma et al., 2001) which suggests unsuccessful proteasomal degradation of αSYN.

Interruption of mitochondria could be another pathogenic role of αSYN. A portion of αSYN is localized within mitochondria (Li et al., 2007a), where it interacts with the mitochondrial

membrane (Nakamura et al., 2008), blocks fusion, and increases its fragmentation (Kamp et al., 2010). Enlargement of mitochondria has also been reported after over-expression of αSYN (Martin et al., 2006; Xie and Chung, 2012). Other reported damage due to mutant or wild type αSYN in mitochondria include impairment of complex I function (Devi et al., 2008; Liu et al., 2009a), mitochondrial membrane potential and ATP production (Banerjee et al., 2010).

αSYN, as well, appears to be involved in oxidative stress-mediated neuronal loss. While over-expression of αSYN hypersensitizes cells to oxidative stress (Tabrizi et al., 2000; Orth et al., 2003) and increases intra-cellular oxidative damage (Lee et al., 2001), its deficiency mediates protection in the MPTP model of dopaminergic death (Dauer et al., 2002; Drolet et al., 2004). Studies demonstrate that oxidative stress and αSYN have more complicated links, where the former can mediate the aggregation of the latter. Accordingly, αSYN interacts with HNE, which is produced by lipid peroxidation under oxidative condition, and this interaction leads to oligomerization and toxicity of αSYN (Qin et al., 2007). In line with this observation, rotenone, an oxidative inducer, reduces αSYN solubility and mediates its aggregation (Sherer et al., 2002).

Together, numerous studies suggest that αSYN pro-death effects are exerted through oxidative stress, and disruption of mitochondrial and proteasomal functions.

1.7.1.2 PARK8 (LRRK2). Leucine-rich repeat kinase 2 (LRRK2) locus (termed PARK8) was identified for the first time in a Japanese family. Patients in this family had relatively late onset and L-dopa-responsive parkinsonism (Funayama et al., 2002). Generally, the clinical symptoms and the pathologic features (such as presence of Lewy bodies) of LRRK2-

linked PD are similar to that of sporadic disease, although the age of onset and the severity could vary from patient to patient.

LRRK2 mutations are known as the most frequent causes of familial PD. More specifically, 5% to 15% of families with a dominant pattern of inheritance and up to 3.6% of sporadic cases have LRRK2 mutations (Funayama et al., 2002; Berg et al., 2005a; Di Fonzo et al., 2005; Paisan-Ruiz et al., 2008). So far, at least seven causative mutations have been reported in this gene, which are all located in highly conserved regions of the encoded protein (Hedrich et al., 2006; Healy et al., 2008). Of these mutations, the most prevalent one is G2019S, which is observed in 7% of inherited (Di Fonzo et al., 2005; Kachergus et al., 2005; Nichols et al., 2005), and up to 2% of sporadic cases (Gilks et al., 2005). The penetrance of PD in the carriers of G2019S mutation at the age of 50-59 years is relatively low (17-28%) (Kachergus et al., 2005; Healy et al., 2008). This rate, however, increases by the age of patients and reaches to almost 80% at the age of 70 years (Kachergus et al., 2005; Healy et al., 2008). Interestingly, an 89 years old G2019S carrier has been reported with no symptoms of PD (Kay et al., 2005), suggesting that the most common pathogenic LRRK2 mutation is not fully penetrant. These reduced penetrance values explicate the relatively common incidence and occasional presence of these mutations in sporadic and control cases respectively (Lesage et al., 2005; Ozelius et al., 2006). This also supports the hypothesis of involvement of several factors and modifiers in development of PD.

The LRRK2 protein consists of 2527 amino acids and several domains. These domains are Ras-of-Complex (ROC) GTPase, C-terminal-of-ROC (COR), serine/threonine protein kinase, ankyrin, leucine-rich repeat motifs, and WD40 repeats (Mata et al., 2006). The physiological quaternary structure of LRRK2 is a dimer, which is necessary for its kinase

activity and membrane localization and appears to be mediated by the ROC and WD40 domains (Deng et al., 2008b; Jorgensen et al., 2009; Sen et al., 2009; Berger et al., 2010). In neurons, it is localized in cytoplasm, in association with membranous and vesicular structures such as the mitochondrial outer membrane and microtubule-associated vesicles (Biskup et al., 2006).

The kinase domain of LRRK2 has a close homology to the mitogen-activated protein kinase kinase kinase (MAPKKK) family. Aalthough several phosphorylation sites and substrates have been reported for this domain in vitro, such as MAP kinase kinase (MKK) proteins (West et al., 2007; Gloeckner et al., 2009; Hsu et al., 2010), α-synuclein (Qing et al., 2009) and β-tubulin (Gillardon, 2009), it shows no evidence of kinase activity in mammalian tissues (Nichols et al., 2009). So far, the most important kinase activity of LRRK2 is believed to be autophosphorylation of several amino acid residues in or close to its GTPase domain (Greggio et al., 2009; Gloeckner et al., 2010). This has been suggested to be an autoregulating mechanism for this protein (Webber et al., 2011). G2019S mutation is located in this domain and appears to elevate the kinase activity of LRRK2 *in vitro* (Smith et al., 2005b; Gloeckner et al., 2006; Moore, 2008).

The GTPase domain of LRRK2 could bind to and hydrolyze GTP *in vitro* (Li et al., 2007b; West et al., 2007). This activity is reduced by R1441C, R1441G, and Y1699C mutations, which are in or close to this domain (Lewis et al., 2007; Li et al., 2007b; Xiong et al., 2010).Whether or not the GTPase activity has a regulatory function on kinase activity is still a subject of controversy (Smith et al., 2006; Ito et al., 2007; West et al., 2007; Taymans et al., 2011). A recent study, however, demonstrated that kinase activity depends on LRRK2 GTP binding capacity but not GTP binding itself (Taymans et al., 2011). These domain-

domain relationships seem even more complicated when considering that, while GTPase activity is independent of the kinase domain (Ito et al., 2007; West et al., 2007), autophosphorylation of the GTPase domain has a regulatory role (Greggio et al., 2009; Kamikawaji et al., 2009; Webber et al., 2011). This explains why mutations in either domain lead to the common result of neuronal loss in PD.

Although there is no consensus on the pathologic function of these mutations, accumulating evidence suggests that these mutations exert toxic effects by causing gain of function in LRRK2. For instance, knocking out LRRK2 does not affect development or survival of dopaminergic neurons (Wang et al., 2008). In addition, over-expression of the wild type, G2019S, or R1441C mutants leads to apoptotic neuronal loss, while disruption of kinase function inhibits cell death (Smith et al., 2005b; Smith et al., 2006; West et al., 2007) (Liu et al., 2008d; Dusonchet et al., 2011; Liu et al., 2011).

LRRK2 protein appears to be involved in several biochemical pathways, including vesicle trafficking, neurite growth, and autophagy. It also controls mitochondrial functions, structures, and dynamics (Biskup et al., 2006; Mortiboys et al., 2010; Wang et al., 2012). In this regard, mutations in LRRK2 promote mitochondrial fragmentation, disrupt ATP synthesis, and elevate ROS production in neurons (Angeles et al., 2011; Wang et al., 2012). These mutants also hyper-sensitize dopaminergic neurons to oxidative agents such as H_2O_2 and paraquat, *in vitro* and in Drosophila models (Imai et al., 2008; Nguyen et al., 2011). A recent study in our lab demonstrated that over-expression of wild type LRRK2 also sensitizes dopaminergic neurons to oxidative stress in Drosophila (Venderova et al., 2009).

1.7.2 Autosomal recessive PD.

1.7.2.1 PARK2 (Parkin). PARK2, located on human chromosome 6, was the first autosomal recessive PD locus identified in adolescent (17-24 years old) parkinsonism. This locus belongs to the Parkin gene (Matsumine et al., 1997; Kitada et al., 1998), whose mutations are responsible for 49% of autosomal recessive PD cases (Lucking et al., 2000) and have been reported in up to 77% of sporadic cases (Lucking et al., 2000). Its encoded protein, Parkin, has 465 amino acids, 50 kDa molecular weight, one ubiquitin-like domain at its N-terminus, two RING finger domains at the C-terminus, and one IBR (in-between ring) domain located between the ring fingers (Beasley et al., 2007). Parkin is localized in cytoplasm, plasma membranes, and mitochondria.

An early age of onset and the absence of Lewy bodies are the main clinical and pathological features differentiating Parkin-linked PD from sporadic PD (Takahashi et al., 1994; Mori et al., 1998; Farrer et al., 2001).

The known physiological function of Parkin is E3 ubiquitin ligase in the proteasomal degradation pathway (Shimura et al., 2000), where its C terminus interacts with E2, another member of proteasome pathway, and its N terminus binds to the proteasome. So far, αSYN, PARIS and synaphilin-1 have been identified as Parkin substrates (Chung et al., 2001; Shimura et al., 2001; Shin et al., 2011). Accumulation of αSYN and synaphilin-1, as a result of Parkin malfunction, leads to formation of Lewy bodies and ubiquitin-positive inclusions in the cytosol (Shimura et al., 2001). PARIS is an expression-suppressor of peroxisome proliferator-activated receptor gamma coactivator-1a (PGC-1α). PGC-1α is a transcription co-activator and has a central role in mitochondrial biogenesis and oxidative metabolism

(Liang and Ward, 2006). Loss of function of Parkin leads to accumulation of PARIS and eventually down-regulation of PGC-1α and its pro-survival pathways (Shin et al., 2011).

Several proteasomal-independent functions have also been reported for Parkin. For instance, regulation of the activity of an acid sensing ion channel (ASIC) (Joch et al., 2007), which is thought to be a protective function as ASIC is involved in excitotoxicity-induced neuronal loss (Pidoplichko and Dani, 2006). Another example involves ubiquitination of Eps15, which eventually leads to activation of the pro-survival AKT/PI3K pathway (Fallon et al., 2006). In addition, Parkin ubiquitinates Hsp70 and suppresses c-Jun N-terminal kinase (JNK) signaling (Liu et al., 2008b), whose pro-death function has been shown in our laboratory in an MPTP model (Hayley et al., 2004).

As mentioned before, the mitochondrion is one of the organelles where Parkin is localized and involved in protection and maintenance. Accordingly, it has been reported that, in mouse and Drosophila models, reduction of Parkin protein level or function leads to mitochondrial damage, elevation of ROS, sensitivity to oxidative stress (Greene et al., 2003; Palacino et al., 2004; Pesah et al., 2004), and disruption of fission and fusion (Deng et al., 2008a). This could be, at least to some extent, through regulation of PARIS and PGC-1α levels, as mentioned above. In addition, Parkin, in association with PINK1, also a PD gene, labels dysfunctional mitochondria to be degraded and turned over through mitophagy (Narendra et al., 2008; Weihofen et al., 2009; Matsuda et al., 2010; Narendra et al., 2010b).

Taken together, these reports suggest an important protective role for Parkin in UPS, mitochondrial dysfunction, and oxidative damage paradigms in neuronal loss.

1.7.2.2 PARK6 (PINK1). PARK6 is located on chromosome 1 (1p36) in humans (Valente et al., 2001) and belongs to PTEN-induced kinase 1 (PINK1) gene (Valente et al., 2001; Valente et al., 2004). PINK1-linked PD is early onset (30-50 years) and its clinical affections resemble those of Parkin-related PD.

PINK1 gene has 8 exons and encodes PINK1 protein, a mitochondrial protein (Valente et al., 2004) with 581 amino acids, a mitochondrial targeting sequence, a transmembrane, and a kinase domain. Most frequent mutations in this gene are missense, although deletion mutations have also been identified (Hatano et al., 2004; Marongiu et al., 2007).

PINK1 belongs to the serine–threonine family of kinases and has several substrates such as Rictor (Murata et al., 2011), tumor necrosis factor receptor associated protein 1 (TRAP1) (Pridgeon et al., 2007), and Parkin. In Drosophila models, PINK1 deficiency-mediated phenotype is similar to that of Parkin deficiency. The phenotype of PINK1 deficiency can be reversed by Parkin expression (Clark et al., 2006; Yang et al., 2006), which suggests that PINK1 acts upstream of Parkin in their biochemical pathway.

Controlling and maintaining mitochondrial functions and dynamics appear to be the main role of PINK1 (Weihofen et al., 2009; Murata et al., 2011). Accordingly, in Drosophila models, PINK1 deficiency leads to an increase of fragmented mitochondrial cristae (Clark et al., 2006) and its over-expression results in induction of mitochondrial fission (Deng et al., 2008a; Poole et al., 2008). In addition, PINK1, in a complex with Parkin, is involved in elimination of dysfunctional mitochondria through mitophagy (Kawajiri et al., 2010; Matsuda et al., 2010). In this regard, PINK1 is localized in the membrane of damaged mitochondria and recruits Parkin to ubiquitinate certain mitochondrial substrates, such as

VDAC1, and induce engulfment of mitochondria by autophagosomes. Functional Parkin, and intact mitochondrial targeting sequence and kinase domain of PINK1 seem to be vital for this process (Narendra et al., 2008; Geisler et al., 2010; Matsuda et al., 2010; Narendra et al., 2010a; Vives-Bauza et al., 2010).

PINK1 also plays a role in oxidative conditions. While knocking down of PINK1 with siRNA hyper-sensitizes neurons to oxidative stress, PINK1 over-expression protects them against this insult (Haque et al., 2008). In line with this report, administration of antioxidants such as SOD1 or vitamin E in PINK1 deficient Drosophila reduces progressive dopaminergic loss (Wang et al., 2006).

1.7.2.3 PARK9 (ATP13A2). PARK9 has been mapped to 1p36 on human chromosome, and the location of the ATP13A2 gene, whose mutations lead to atypical PD with an autosomal recessive pattern of hereditary. The ATP13A2-mediated PD is termed Kufor-Rakeb syndrome (Ramirez et al., 2006), and is characterized by very early onset (14-16 years old), fast progression, dementia, and supranuclear gaze palsy.

The ATP13A2 gene is composed of 29 exons and encodes a lysosomal membrane protein with 1180 amino acids, one ATPase, and ten transmembrane domains (Ramirez et al., 2006). The identified pathogenic mutations have direct or indirect destructive effects on the transmembrane domains, most of which generate unstable truncated forms of the protein that are not localized in the lysosomal membrane.

ATP13A2 protein is a P5-type ATPase that is involved in controlling cation homeostasis and preventing toxicity induced by manganese and α-synuclein (Gitler et al., 2009). Wild-type ATP13A2 also inhibits release of cytochrome C into the cytoplasm (Tan et al., 2011).

1.8 PARK7 (DJ-1)

DJ-1 mutations have been identified in 1-2% of familial PD cases (Pankratz et al., 2006). These mutations lead to an autosomal recessive form of PD with clinical symptoms similar to those of Parkin and PINK1-related PD.

DJ-1 is a conserved gene across species, which contains eight exons and encodes a 189-amino acid protein with 20-25 kDa molecular weight, a single folded domain, dimeric structure and ubiquitous expression(Macedo et al., 2003; Wilson et al., 2003). This protein has been reported as an oncogene with transforming capability, specially associated with H-ras (Nagakubo et al., 1997), a regulatory subunit of an RNA-binding complex (Hod et al., 1999), and an androgen receptor-regulating protein (Niki et al., 2003; Pitkanen-Arsiola et al., 2006). Its role has also been demonstrated in diseases such as amyotrophic lateral sclerosis (ALS) (Annesi et al., 2005; Lev et al., 2009) and several cancers (Hod, 2004; Davidson et al., 2008; Kim et al., 2009; Miyajima et al., 2010). The notion that it is involved in a wide variety of diseases reflects its complex and multifunctional nature. Examples of these potential functions are chaperone activity (Lee et al., 2003; Shendelman et al., 2004), RNA binding activity (van der Brug et al., 2008; Blackinton et al., 2009b), protease function (Koide-Yoshida et al., 2007; Chen et al., 2010), and transcriptional co-activation (Clements et al., 2006; Zhong et al., 2006). There are also several reports indicating its interaction with different proteins and cellular pathways such as Daxx (Junn et al., 2005), an apoptosis-related protein, ASK1 (Im et al., 2010; Mo et al., 2010), an apoptosis regulating kinasem, p53 (Bretaud et al., 2007; Fan et al., 2008b; Fan et al., 2008a), and sumoylation-related proteins (Shinbo et al., 2006; Fan et al., 2008b). The mechanisms of function of DJ-1 on

these proteins and its involvement in the related biochemical pathways, however, are the subjects of further studies.

Since 2003, when Bonifati and his colleagues identified DJ-1 as the gene associated with PARK7 in an Italian family with an early onset PD (Bonifati et al., 2003), many researchers have drawn their attention to this protein as a new subject of study in PD research. As DJ-1 is the main focus of this dissertation, the structure, mutations and proposed functions of this protein in PD will be reviewed, although briefly, in the following sections.

1.8.1 Molecular structure of DJ-1. DJ-1 is a small protein with seven β-strands, nine α-helices, a flavodoxin-like Rossmann fold and a globular homodimer quaternary structure in both physiological and crystal phases (Honbou et al., 2003; Huai et al., 2003; Lee et al., 2003; Tao and Tong, 2003; Wilson et al., 2003). This structure appears to be important in DJ-1's biological function as its disturbance leads to pathogenesis. For instance, one of its pathogenic mutants, L166P, occurs in α-helix G (Honbou et al., 2003; Huai et al., 2003; Lee et al., 2003; Tao and Tong, 2003; Wilson et al., 2003) and leads to interruption of dimeric structure, protein degradation through both proteasomal and non-proteasomal systems, and ultimately reduction of its level in the cell (Miller et al., 2003; Moore et al., 2003; Gorner et al., 2004). Unlike the L166P mutation, other pathogenic mutations do not lead to dramatic changes in DJ-1's structure, suggesting that even slight deformities in its molecular structure could have significant loss of function effects (Hering et al., 2004; Lakshminarasimhan et al., 2008; Malgieri and Eliezer, 2008).

DJ-1 contains three cysteine residues at positions 46, 53 and 106. Cys106 is highly conserved, appears to have prominent reactivity (Wilson et al., 2003), and seems to be the

most essential cysteine for DJ-1 function. This residue is located at the lowermost part of the surface groove, where a β-strand connects to an α-helix with a deep turn (Honbou et al., 2003; Huai et al., 2003; Lee et al., 2003; Tao and Tong, 2003; Wilson et al., 2003). Although the physical position and ionization features of Cys106, such as low pK_a value, stabilization of the Cys106-SO_2^- by Glu18 (Canet-Aviles et al., 2004; Blackinton et al., 2009a), proximity of an active SO_2^- to a protonated glutamic acid, and perturbed ionization profile of amino acids around this residue (Wei et al., 2007), make it a potential candidate for an enzymatic active site, a recent study ruled out this hypothesis. In this study it was demonstrated that a C106DD mutant of DJ-1 and wild type DJ-1 were almost equally able to protect cells under oxidative conditions (Waak et al., 2009), although the C106DD mutant does not have the nucleophilic thiol group that is hypothetically essential for the suggested enzymatic functions.

1.8.2 DJ-1 mutations. Pathogenic mutants of any disease-related gene are, indeed, important tools for studying the structural and functional deformities of the encoded proteins, which usually lead to activity impairments and finally pathogenesis. In the case of DJ-1, several mutations have been reported in both genetic and sporadic PD. The potential destructive impact of these mutations on DJ-1 function in the context of PD pathogenesis is the subject of numerous studies. These mutations are summarized in table 1.

Mutation	Reported inheritance	Location on the gene	Reported population	Potential effects	References
L166P	Homozygous	Exon 7	Italian	Interruption of homodimer structure, increase of protein turnover, and decease of protein stability	(Bonifati et al., 2003; Macedo et al., 2003; Tao and Tong, 2003; Gorner et al., 2004)
14 kb deletion	Homozygous	4kb upstream of the open reading frame to exon 5	Dutch	Protein loss	(van Duijn et al., 2001; Bonifati et al., 2003)
M26I	Homozygous	Exon 2	Ashkenazi Jewish	Decrease of dimerization and stability, but milder than that of L166P	(Abou-Sleiman et al., 2003; Blackinton et al., 2005)
E64D	Homozygous		Turkish	Decrease of flexibility, Unknown effect on function	(Abou-Sleiman et al., 2003; Blackinton et al., 2005)
E163K	Homozygous	Exon 7	Italian	Unknown effect	(Annesi et al., 2005)
A104T	Heterozygous	Exon 5	Asian	Unknown effect	(Clark et al., 2004; Blackinton et al., 2005)
D149A	Heterozygous	Exon 7	Afro-Caribbean	Decrease of thermo-stability and nuclear localization, Unknown effect on its function	(Abou-Sleiman et al., 2003; Xu et al., 2005; Malgieri and Eliezer, 2008)

Table 1: DJ-1 mutations

1.8.3 DJ-1, oxidative stress, and mitochondria. As DJ-1 is expressed ubiquitously, it is not possible to hypothesize a role for it based on its tissue-specific pattern of expression. In addition, its involvement in several fields/diseases such as oncogenesis and male fertility and also its potential to interact with RNA (Hod et al., 1999; van der Brug et al., 2008) and numerous proteins (Junn et al., 2005; Li et al., 2005; Jin et al., 2007; Mo et al., 2008), make it difficult to consider a single function for this protein. In this introduction, however, we will focus on the role of DJ-1 in protecting neurons or other cell types from mitochondrial and oxidative damage, which is supported by many studies.

A growing body of evidence supports the protective role of DJ-1 against oxidative stress (Martinat et al., 2004; Taira et al., 2004; Kim et al., 2005a; Aleyasin et al., 2007; Aleyasin et al., 2010). Accordingly, in our lab we have demonstrated that DJ-1 deficiency leads to hyper-sensitivity of dopaminergic neurons to oxidative stress in both *in vivo* and *in vitro* models. This hyper-sensitivity can be reversed by re-introduction of DJ-1 to these cells (Kim et al., 2005a). In addition, our data show higher levels of oxidative damage in DJ-1 deficient neurons compared to wild type neurons after an ischemic insult (Aleyasin et al., 2007). The response of DJ-1 to oxidative stress includes decrease of its isoelectric pH (Mitsumoto and Nakagawa, 2001) and translocation of the oxidized form to the mitochondrial outer membrane (Canet-Aviles et al., 2004). Cys 106 appears to play a critical role in these actions, as its replacement with alanine, serine, or aspartic acid disrupts the translocation and protective effects of the protein in oxidative stress (Kinumi et al., 2004; Aleyasin et al., 2007; Blackinton et al., 2009a; Kim et al., 2009; Waak et al., 2009; Im et al., 2010; Giaime et al., 2012; Joselin et al., 2012). Interestingly, even in yeast and bacteria, DJ-1 homologs appear to possess the protective functions in the same paradigm, where its expression is

induced in response to oxidative stress, and its impairment sensitizes cells to this condition (de Nobel et al., 2001; Abdallah et al., 2007). The structurally equivalent cysteine of human Cys 106 in these homologs is also easily oxidized to Cys106-SO_2^- (Wilson et al., 2004; Wilson et al., 2005), suggesting that it acts with the same mechanism as mammalian DJ-1.

Translocation of DJ-1 to mitochondria in certain conditions such as exposure to mitochondrial toxins MPTP and MPP^+ (Canet-Aviles et al., 2004; Blackinton et al., 2005; Lev et al., 2008; Joselin et al., 2012) suggests a regulatory or protective role for this protein in mitochondrial maintenance and function. In line with this hypothesis, DJ-1 has a protective effect against MPTP and MPP^+ (Canet-Aviles et al., 2004; Taira et al., 2004; Kim et al., 2005a). While the protective function of DJ-1 is abolished by inhibition of its translocation to mitochondria (Canet-Aviles et al., 2004), targeting this protein to mitochondria results in significantly higher levels of protection against oxidative stress (Junn et al., 2009). Several reports indicate that DJ-1 also has roles in biological functions and dynamics of mitochondria. For instance, DJ-1 deficiency leads to decrease of the activity of mitochondrial complexes (Ooe et al., 2005; Andres-Mateos et al., 2007; Hayashi et al., 2009; Heo et al., 2012), increase of mitochondrial ROS production, and induction of mitochondrial fragmentation and morphology disturbances (Blackinton et al., 2009a; Irrcher et al., 2010; Krebiehl et al., 2010; Giaime et al., 2012). Increased mitochondrial fragmentation and morphology defects have been confirmed in DJ-1-linked PD lymphoblasts by our laboratory (Irrcher et al., 2010). In addition, DJ-1 induces the transcriptional function of PGC-1α, which as mentioned before, is a protein with a critical role in mitochondrial biogenesis (Zhong and Xu, 2008).

Taken together, data obtained from numerous studies indicate that DJ-1 possesses an important role in handling the oxidative load of the cells and protecting the function, morphology, and dynamics of mitochondria. While Cys 106 seems to be involved in these processes, the potential causative link between oxidative stress-induced modifications of this residue and DJ-1 protective function is yet to be elucidated.

1.8.4 The proposed mechanisms of function of DJ-1. Although the outcome of most studies is that DJ-1 protects neurons against oxidative stress, the details and the mechanism(s) of this action have not been fully revealed. Here, we summarize the proposed mechanisms through which DJ-1 might exert its protective function.

1.8.4.1 ROS scavenging. DJ-1 is classified as an atypical peroxy-redoxing protein because it possesses H_2O_2 scavenging ability and a reactive cysteine, which is able to shift to Cys-SO_2H (Taira et al., 2004). This activity, however, appears to be too weak to explain DJ-1's potentials in handling oxidative stress and protecting neurons under such conditions (Yang et al., 2005; Andres-Mateos et al., 2007). As a result, other hypotheses (below) were considered to figure out the mechanism of function of DJ-1.

1.8.4.2 Regulation of anti oxidant gene/protein expression. DJ-1 appears to have a regulatory role in expression of a number of genes at different levels. For instance, DJ-1 interacts with mRNA of certain genes and inhibits their translation. Oxidation of DJ-1 in response to oxidative insult disrupts this interaction and results in activation of protein synthesis (van der Brug et al., 2008). Regulation of GPx4 and MAPK8IP1 by DJ-1 through this mechanism has been reported in PD post mortem tissues (Blackinton et al., 2009b).

DJ-1 can also regulate gene expression by exerting/inhibiting post-translational

modifications of certain proteins. For instance, PIASx-alpha inhibits androgen receptor transcriptional activity by interacting with it. This inhibitory function is abolished by interaction of DJ-1 with PIASx-alpha and retracting it from the androgen receptor-PIASx-alpha complex (Takahashi et al., 2001). Another example involves regulation of expression of MnSOD, an antioxidant protein, by DJ-1. In this case, transcription of MnSOD is activated by PGC-1α, whose activity is suppressed by PSF. DJ-1 reduces inhibitory function of PSF by inhibiting its SUMOylation. The outcome of DJ-1 post-translational function on PSF is induction of expression of an antioxidant enzyme (MnSOD) (Zhong and Xu, 2008). Inhibition of PSF by DJ-1 through this mechanism also leads to induction of tyrosine hydroxylase expression (Zhong et al., 2006).

Another example includes induction of synthesis of glutathione, a well known antioxidant protein, by DJ-1 through increasing glutamate cysteine ligase (Zhou and Freed, 2005). DJ-1 can also stabilize Nrf2, an antioxidant transcriptional activator, in MEFs (Clements et al., 2006). This could potentially induce expression of several antioxidant proteins and provide protection.

1.8.4.3 Chaperone activity. Unfolded protein response is another area where DJ-1's function has been reported. Its close homology to heat shock proteins and a family of bacterial proteases initially suggested this function for DJ-1 (Lee et al., 2003). In addition to this structural relevance, the chaperone-like activity of DJ-1 has also been reported, where, in an oxidative sensitive manner, it suppresses accumulation of α-SYN in cytoplasm (Shendelman et al., 2004; Zhou et al., 2006). Oxidation of Cys 106 to some median levels (Cys-SO_2-H) seems to be essential for this activity (Zhou et al., 2006). DJ-1 also prevents aggregation of microtubule associated protein 1 B (MAP1B) through its chaperone activity (Wang et al.,

2011).

1.8.4.4 Regulation of pro-death and pro-survival signaling. In addition to its chaperone function or post-translational and post-transcriptional modulating activity, DJ-1 is involved in cell signaling in favour of survival pathways. For instance, DJ-1 inhibits interaction of ASK-1 and Daxx. Translocation of Daxx from nucleus to cytoplasm and its interaction with ASK-1 is essential for activation of ASK-1 and downstream pro-apoptotic pathways. In response to oxidative insult, DJ-1 sequesters Daxx in the nucleus and inhibits the apoptotic cascade (Junn et al., 2005).

DJ-1 also positively regulates some pro-survival signalling, such as the AKT/PIP3 pathway (Sitaram et al., 2009; Vasseur et al., 2009; Aleyasin et al., 2010; Yao et al., 2011). AKT is a kinase with neuro-protective properties under multiple conditions, including oxidative stress (Dudek et al., 1997; Lee et al., 2009b; Sun et al., 2010), which is activated by interacting with a membrane phospholipid, PIP3 (Kohn et al., 1996). In our laboratory, we have shown that in response to MPTP/MPP^+-mediated oxidative stress, DJ-1 modulates AKT recruitment to the membrane and PIP3 and thus activates the protective function of AKT (Aleyasin et al., 2010).

Taken together, the accumulating body of evidence from *in vitro* and *in vivo* studies indicates that DJ-1, a single domain protein, is involved in a number of pathways to provide resistance against oxidative and mitochondrial stress in cells. One of the most important areas of controversy, which could provide invaluable insights in understanding the mechanisms underlying neuronal loss in PD and potential therapeutic strategies, is the mechanism(s) by which DJ-1 provides this protection. Hence, it is of critical importance to uncover the

biochemical partners and interacting proteins of DJ-1.

Previously, in an attempt to create a map demonstrating human protein-protein interactions, our collaborator Dr. Daniel Figeys and his colleagues, at the Department of Biochemistry, Microbiology and Immunology (BMI) of the University of Ottawa, performed a mass proteomic interaction study (Ewing et al., 2007). In this study, the authors selected 407 human proteins as baits, based on their roles in diseases such as cancers, obesity and diabetes, and used them to immunoprecipitate possible interacting proteins from human cell lysate. The isolated proteins were then identified by mass spectrometry.

With the goal of identifying DJ-1 interactors, we analyzed the resulting data focussing on DJ-1. The results of peptide analysis showed that DJ-1 was immunoprecipitated with two novel bait proteins, Paraoxonase2 (PON2) and Von Hipple Linau (VHL) protein.

1.9 Paraoxonase family

Paraoxonase2 (PON2) is a member of the Paraoxonase family with two other members, PON1 and PON3. PON genes are clustered on chromosome 7 in humans, and share almost 70% homology in nucleotide sequence.

1.9.1 PON1 and PON3. The PON1 gene has nine exons and encodes an ~45kDa glycoprotein with calcium-dependent esterase activity and the ability to breakdown organophosphates, lactones and paraoxons (Davies et al., 1996; Billecke et al., 2000; Jakubowski, 2000; Draganov and La Du, 2004). Although it is expressed in several tissues, its main tissue of expression is liver, from which it is secreted into the blood and carried by HDL (La Du, 1996; Marsillach et al., 2008). Numerous studies have indicated that PON1 is

an antioxidant and protects against oxidation of LDL, generation of macrophage foam cells, and atherosclerosis (Shih et al., 2000; Tward et al., 2002; Ng et al., 2005; Mackness et al., 2006; Coombes et al., 2011; Precourt et al., 2011; Rosenblat et al., 2011; Bayrak et al., 2012). Interestingly, its gene polymorphisms have associations with several physiological complications such as coronary heart disease (Ito et al., 2002), diabetes (Hofer et al., 2006), and, of most interest to this study, PD (Zintzaras and Hadjigeorgiou, 2004).

The PON3 gene also has nine exons, and its 40 kDa protein is expressed mostly in liver and kidneys, secreted into serum circulation, and binds to HDL in humans. Like PON1, PON3 has antioxidant properties, where it decreases LDL oxidation (although to a lower extent than PON1) , inactivates oxidized LDL, decreases lipid hydroperoxidation and monocyte chemotaxis, decreases oxidative marker malondialdehyde (MDA), and increases glutathione level and super oxide dismutase (SOD) activity (Reddy et al., 2001; Liu et al., 2008c; Peng et al., 2009; Kempster et al., 2012). Transgenic mice over-expressing human PON3 and kept on an atherogenic diet showed lower levels of obesity and atherosclerosis than controls (Shih et al., 2007). Other studies also confirm PON3's anti-atherosclerosis property (Ng et al., 2007). While two missense mutations have been reported in PON3 (Campo et al., 2004), their effects in human disease have yet to be studied.

Taken together, most studies indicate an anti-atherosclerotic function for PON1 and PON3, which is believed to be mediated through their antioxidant properties.

1.9.2 PON2. Evolutionarily, PON2 seems to be the oldest member of PON family (Primo-Parmo et al., 1996; Draganov and La Du, 2004; Ng et al., 2005). Its gene, like other two members, has nine exons, which encode a 42-44 kDa protein. This protein is intra-cellular,

mostly associated with membranes, and is not released into serum (Ng et al., 2001; Li et al., 2003; Ng et al., 2005). It does not have any detectable paraoxonase or organophosphatase activity, but can hydrolyse lactones. So far, its physiological roles have been reported to be handling of oxidative stress and protection against inflammation and atherosclerosis. Unlike PON1 and PON3, PON2 expression is ubiquitous (Ng et al., 2001; Li et al., 2003; Ng et al., 2005).

1.9.2.1 PON2, oxidative stress, ER stress and inflammation. The antioxidant activity of PON2 was reported for the first time by Ng and colleges, where over-expression of PON2 in HELA cells led to reduction of intracellular ROS and oxidation of LDL exposed to these cells (Ng et al., 2001). They also showed that PON2 has the ability to reverse the effects of pre-oxidized LDL, and unlike PON1 and PON3, PON2 has antioxidant effects at the cellular level. This role of PON2 was also demonstrated in vascular cells over-expressing PON2 (Horke et al., 2007). These cells showed lower levels of ROS after induction of oxidative stress compared to cells with no PON2 over-expression. In this study, it was also demonstrated that PON2 is protective against ER stress. This, along with the presence of an ER stress-like element in the promoter of PON2, suggests an important role of PON2 in managing oxidative damage and ER stress in vascular cells. Anti-ER stress feature of PON2 has also been demonstrated in macrophages (Devarajan et al., 2012).

Studies on PON2 and apo E deficient mice revealed the anti-atherosclerosis role of PON2 (Ng et al., 2006b; Ng et al., 2006a). These studies demonstrated that PON2 deficient mice are more prone to develop atherosclerosis and have higher levels of LDL oxidation and monocyte chemotaxis. In line with this result, while apo E deficiency increases the risk of atherosclerosis in mice, over-expression of PON2 reduces this risk (Ng et al., 2006a). In

addition, macrophages in PON2 deficient mice also showed elevated TNF-α and IL-1β expression, suggestive of higher susceptibility to inflammation responses in the absence of PON2 (Ng et al., 2006b). Increased oxidative level, inflammation-inducing modifications of LDL, and inflammatory responses from macrophages are thought to be the main reasons for the increased risk of atherosclerosis in PON2 deficient mice. The inhibition of oxidative stress in macrophages by PON2 is also suggested in other studies. In these studies it has been demonstrated that under high glucose conditions, PON2 reduces macrophage triglyceride synthesis and accumulation and macrophage oxidative stress by reduction of NADPH-oxidase and diacylglycerol acyltransferase1 (DGAT1) activity (Rosenblat et al., 2009; Meilin et al., 2010). This demonstrates the protective role of PON2 against pathogenic functions of macrophages in atherosclerosis and vascular disorders in diabetes. In line with these observations, purified PON2 has also been shown to have antioxidant function in macrophages (Rosenblat et al., 2003).

In a recent study, Precourt and colleagues showed that knocking down of PON2 in Caco-2/15 cells, leads to disturbances in levels of superoxide dismutases and catalase, increase of hydrogen peroxide and MDA and decrease of reduced to oxidized glutathione ratio, suggesting the antioxidant function of this protein in intestinal cells as well (Precourt et al., 2012).

Interestingly, it has been demonstrated that inhibition of PON2 expression reduces the antioxidative effects of polyphenol compounds in pomegranate juice (Shiner et al., 2007). The authors conclude that the pomegranate juice antioxidants exert their antioxidative effects, to some extent, by induction of PON2 expression and activity.

Another novel function of PON2 is its high potential to breakdown acylhomoserine lactones, an important pro-infection agent involved in bacterial communication, activity, and pathogenic function (Juhas et al., 2005). The process mediated with this lactone, termed quorum sensing, is up-regulated in PON2 deficient mice (Stoltz et al., 2007). Interestingly, PON2 is the most effective member of the PON family in inhibiting quorum sensing (Draganov et al., 2005), making it a good candidate for therapeutic strategies against bacterial infections.

Pathophysiological conditions associated with inflammation and oxidative stress in several cell types and models appear to be the effective inducers of PON2 activity/ expression (Rosenblat et al., 2003; Shiner et al., 2004; Fuhrman et al., 2008). For instance, PON2 mRNA and lactonase activity are up-regulated in mouse peritoneal macrophages (MPM) in response to apo E deficiency and oxidative stress induced by buthionine sulfomixine, angiotensin II, and ox-PAPC (Rosenblat et al., 2003). Similar responses were also observed in human monocytes during their differentiation to macrophages, a process associated with increased superoxide production (Shiner et al., 2004), or in THP-1 macrophages after treatment with urokinase plasminogen activator (uPA), an inducer of oxidative stress (Fuhrman et al., 2008), and in J774 A.1 macrophages in response to copper sulfate and iron/ascorbate treatment (Shiner et al., 2006). This induction of PON2 lactonase activity and expression is oxidative dependent, as inhibitors of NADPH-oxidase inhibit this response (Shiner et al., 2004).

Taken together, evidence collected from PON2 studies shows the strong protective function of this protein from oxidative stress, ER stress, and inflammation. While these protective roles are exerted at the cellular level in almost all tissues, the protection by PON1 and PON3

appears to be limited to blood circulation. These protective roles alone could make PON2 a target for PD research, and our finding that DJ-1 is a biochemical partner of PON2 makes it even more interesting.

1.10 Von Hipple Lindau (VHL)

The VHL gene is primarily a tumor suppressor with 3 exons and ubiquitous expression. Mutations in this gene cause familial VHL disease (Latif et al., 1993), which gives rise to tumorigenesis in several organs (Maher et al., 2011), primarily kidneys as clear-cell renal cell carcinoma (ccRCC), and the central nervous system as hemangioblastoma.

The VHL gene encodes two protein products (pVHL30 and pVHL19), which are both termed VHL protein or pVHL and biochemically act similarly. VHL protein is mostly cytoplasmic but could also be localized in the nucleus, mitochondria, and ER (Kaelin, 2002). Its translocation between cytoplasm and nucleus appears to be crucial in its tumor suppressing function (Lee et al., 1996; Lee et al., 1999).

1.10.1 VHL functions. Through protein-protein interactions and gene expression studies, multiple physiological functions have been identified for VHL protein. These functions include:

Regulation of apoptotic pathways. VHL protein has been reported to be involved in regulation of p53 function via inhibition of its MDM2-mediated ubiquitination and nuclear translocation, and eventually enhancing its transcriptional activity (Roe et al., 2006). Thus, as a result of VHL deficiency, p53 and its downstream apoptotic pathways will be inactivated. Furthermore, VHL inactivates NF-κB function by acting as an adaptor that

mediates the phosphorylation and inhibition of NF-κB agonist, CARD9 (Yang et al., 2007). Given that NF-κB acts as an anti-apoptotic agent in cancer cells (Sheikh and Huang, 2003), VHL loss could lead to activation of this protein and its anti-apoptotic cascade.

Stabilization of microtubules. VHL protein interacts with and stabilizes microtubules, a function lost in VHL mutations that leads to development of haemangioblastomas (Hergovich et al., 2003). This function of VHL is crucial in maintenance of the primary cilium in several tissues, as the ciliary axoneme basically possesses a microtubular structure. The primary cilium is an extracellular structure acting as a sensory device of the cell to follow and modulate the chemical and mechanical signals (Singla and Reiter, 2006). Loss of ciliogenesis and function is associated with development of renal cysts, VHL disease, polycystic kidney disease, and Bardet Biedl syndrome (Siroky and Guay-Woodford, 2006).

Formation of extracellular matrix. Wild type VHL, but not mutated forms of this protein, associates with fibronectin and hydroxylated collagen IV (Hoffman et al., 2001; Kurban et al., 2008), components of extracellular matrix. Loss of this interaction leads to dysfunctional deposition of extracellular matrix (Ohh et al., 1998; Bishop et al., 2004; Tang et al., 2006). In addition, formation of adherent and tight junctions is also disrupted in VHL deficient cells (Calzada et al., 2006). Intact extracellular matrix is an important barricade against metastasis of cancer cells and an important factor in preserving cell polarity (Tsukita et al., 2001). How VHL, an intracellular protein, regulates formation of extracellular structures is yet to be elucidated.

E3 ubiquitin ligase and regulation of HIF-1α stability. The best known and most studied function of VHL protein is the substrate recognition activity in an E3 ubiquitin ligase

complex composed of Cullin-2, elongin B and C, and Rbx-1 (Pause et al., 1997; Lonergan et al., 1998; Kamura et al., 1999; Lisztwan et al., 1999; Stebbins et al., 1999). The VHL target protein for ubiquitination is the unstable subunit of hypoxia inducible factor 1 (HIF-1), termed α subunit or HIF-1α.

HIF-1 is a transcription factor responsible for initial and immediate transcriptional responses to hypoxic and oxidative stresses. This transcription factor is a basic helix-loop-helix protein, composed of two subunits, α and β. These subunits, when they bind together and make the active heterodimer transcription factor, interact with hypoxia-responsive elements (HREs) of the target genes and activate their transcription. The β subunit is stable and constitutively expressed and active. The α subunit, however, with an oxygen dependent degradation (ODD) domain, is unstable and, under physiological conditions, degraded by proteasome. Under normal conditions (normoxia), the α subunit is hydroxylated on its proline residues, which reside in the ODD domain, by prolyl hydroxylases (PHD) (Bruick and McKnight, 2001; Ivan et al., 2001; Jaakkola et al., 2001; Yu et al., 2001a). The hydroxylated prolines are targets of VHL, which interacts with them and mediates ubiquitination and degradation of HIF-1α by E3 ligase and proteasomes, respectively (Masson et al., 2001; Yu et al., 2001b). Under hypoxic conditions, PHD is inhibited, HIF-1α is not ubiquitinated by VHL and degraded by proteasomes, and is stabilized. This activates transcription of rescue genes such as erythropoietin (EPO) and vascular endothelial growth factor (VEGF) (Figure1.2).

There are several lines of evidence showing that ROS is crucial in hypoxic response of HIF-1α . First, hypoxia induces ROS generation in the cells. Second, antioxidants, such as glutathione peroxidase or pyrrolidine dithiocarbamate, inhibit stabilization of HIF-1α

induced by hypoxia. Third, induction of H_2O_2 production in the cells or application of exogenous H_2O_2 could stabilize HIF-1α, independent of hypoxia (Chandel et al., 1998; Chandel et al., 2000; Richard et al., 2000; Gerald et al., 2004; Brunelle et al., 2005; Simon, 2006; Liu et al., 2008a). These findings suggest that hypoxia inhibits PHD and stabilizes HIF-1α via ROS production. The role of ROS in this paradigm would in turn suggest VHL/HIF-1 pathway as an important player in PD pathogenesis or at least in PD models.

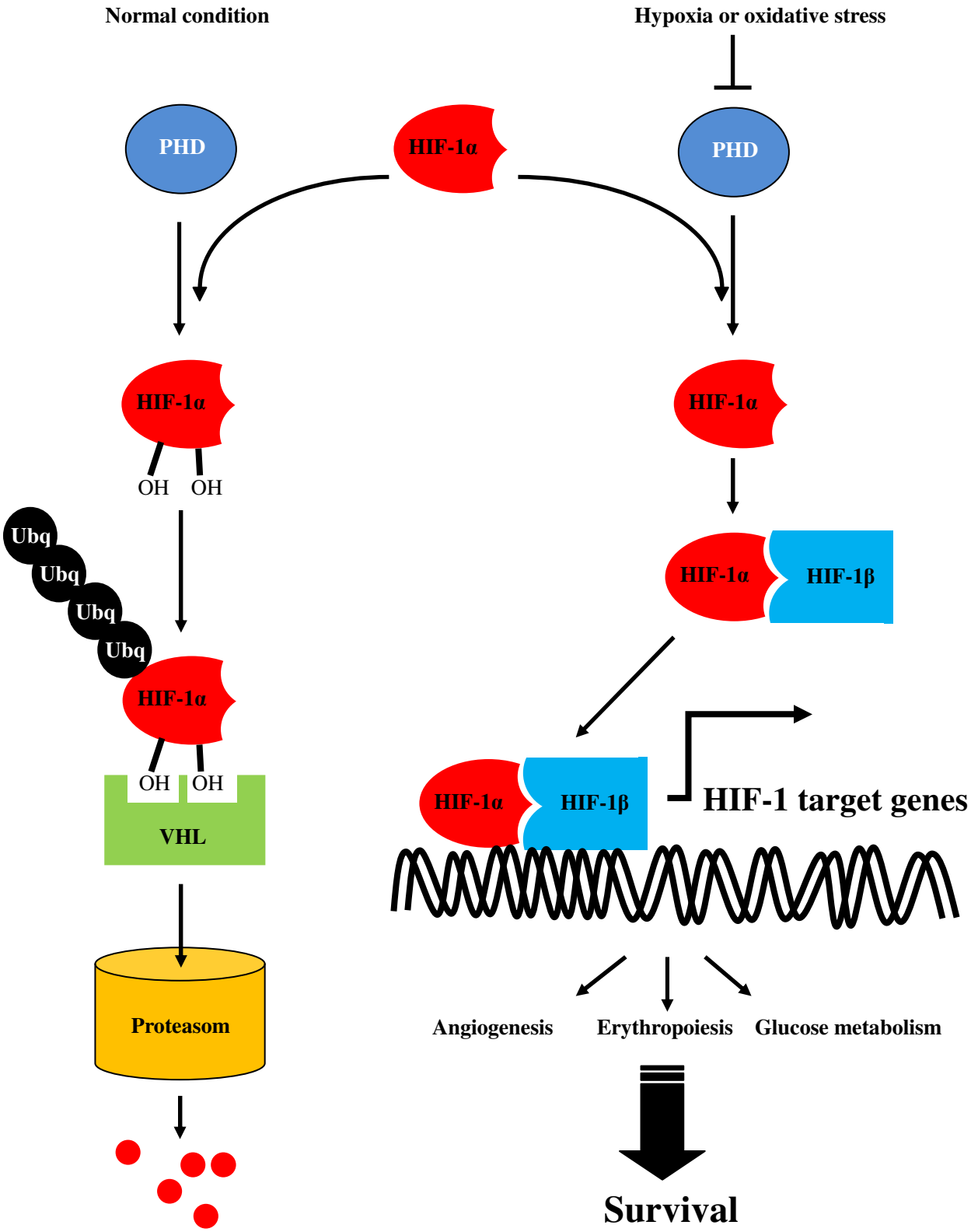


Figure 1.2: VHL/ HIF-1α pathway

Figure 1.2: At normal conditions, HIF-1α is hydroxylated by Prolyl hydroxylase (PHD), poly-ubiquitinated by VHL and degraded by proteasom. In response to hypoxia or oxidative stress, HIF-1α hydroxylation by PHD and subsequent poly-ubiquitination by VHL are inhibited. This results in stabilization and activation of HIF-1, transcription of its target genes, and induction of their downstream pathways such as angiogenesis, erythropoiesis and glucose metabolism.

1.10.2 VHL/HIF-1 pathway and PD. The growing body of evidence suggesting the involvement of HIF-1 in PD is notable. First, analysis of alterations of gene expression in PD SNc revealed a down-regulation of HIF-1 signaling in these cases (Elstner et al., 2011). Second, up-regulation of HIF-1 or its target genes has widely been reported to be protective against *in vivo* and *in vitro* models of PD (Genc et al., 2001; Genc et al., 2002; Siddiq et al., 2005; McLeod et al., 2006; Wu et al., 2007c; Wu et al., 2007b; Lee et al., 2009a; Johansen et al., 2010; Wu et al., 2010; Cui et al., 2011). For instance, inhibition of PHD in mouse, with administration of 3,4-dihydroxybenzoate (DHB), besides elevating the levels of HIF-1, protects dopaminergic neurons against MPTP and rescues MPTP-mediated reduction in pyruvate dehydrogenase mRNA and activity (Lee et al., 2009a). Administration of EPO into SNc can also restore locomotor activities of MPTP-treated mice, and almost fully protect SNc dopaminergic neurons (Genc et al., 2001). Interestingly, VEGF, in addition to exerting a protective role against 6-OHDA in SNc and striatum of rats, can also rescue already damaged striatum tissues 2 weeks after 6-OHDA treatment (Yasuhara et al., 2005). Third, HIF-1 induces expression of tyrosine hydroxylase, one of the key enzymes in dopamine synthesis, and its accumulation leads to elevation of dopamine release in rat ventral mesencephalic cells (Johansen et al., 2010).

These links of HIF-1 to PD, together with our initial finding that VHL and DJ-1 are potential biochemical partners, provide a cue to investigate the functional effect of DJ-1 on VHL and the HIF-1 pathway.

1.11 Statement of research questions, hypothesis and objectives.

As mentioned before, in the context of PD, DJ-1 is neuro-protective against oxidative stress. To reach a better insight towards the mechanisms of function of this protein, it is important to explore its biochemical partners, study the potential effects of DJ-1 on these proteins, and examine the potential changes in their functions in DJ-1 deficient neurons.

Based on the protective role of DJ-1, PON2, and VHL downstream protein, HIF-1, in oxidative insults, and our initial observation that DJ-1 interacts with PON2 and VHL, we hypothesize that DJ-1 exerts its protective function through inhibiting VHL activity and enhancing HIF-1 stability, and also through inducing PON2 pro-survival activity (Figure1.3).

To address these goals, the following objectives were proposed in this research project:

Objective 1: To confirm the potential interaction between DJ-1 and VHL, and also between DJ-1 and PON2.

Objective 2: To determine the effects of DJ-1 on function/ protein level of each partner, and examine the possible modifications occur in expression/function of these proteins in DJ-1 deficient neurons.

Objective 3: To examine the significance of each protein in neuronal survival in an *in vitro* model of PD.

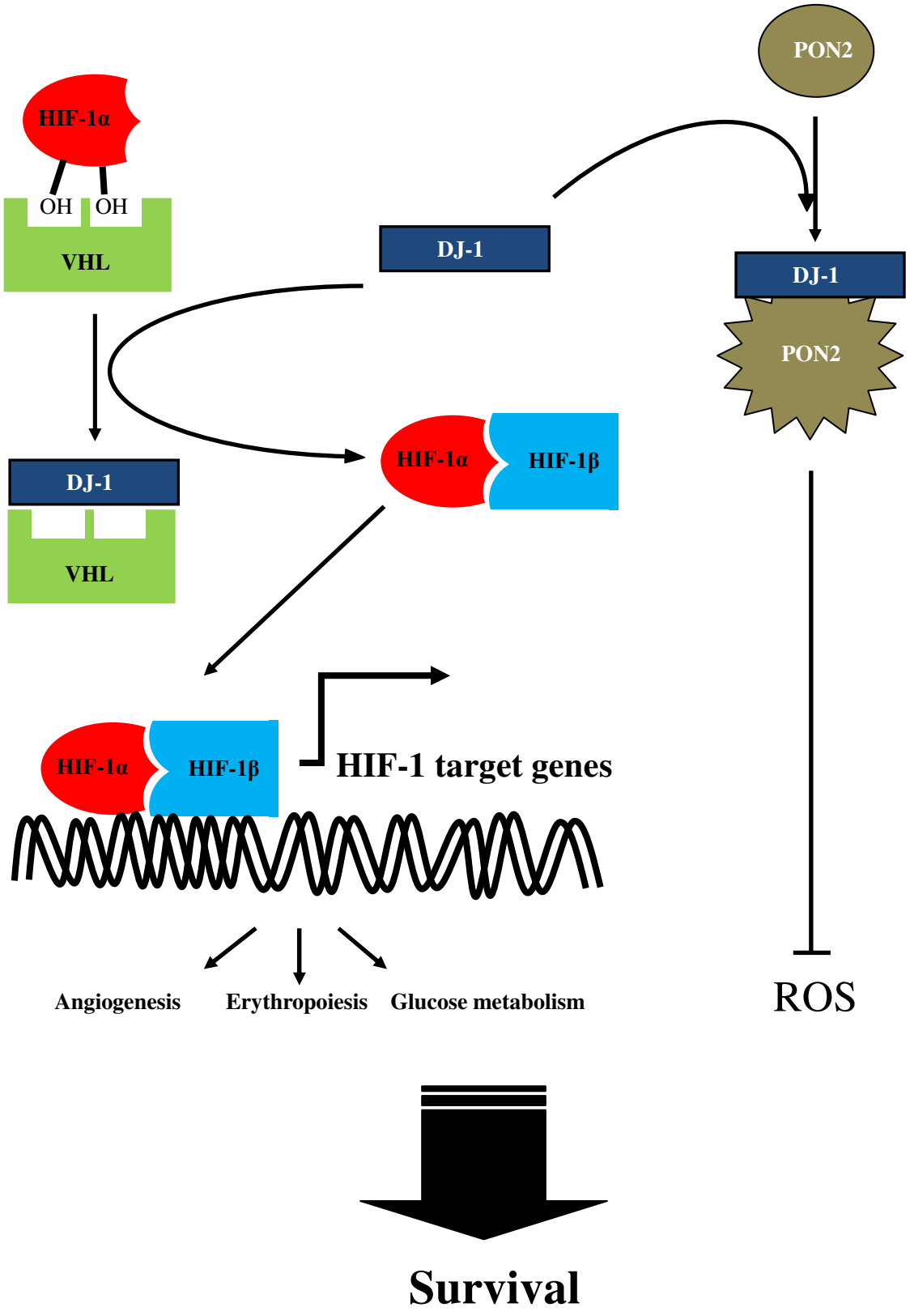


Figure 1.3: Hypothesis

CHAPTER 2

DJ-1 interacts with and regulates PON2, an enzyme critical for neuronal survival in response to oxidative stress

Statement of authors contribution

The research project presented in this manuscript was performed in cortical neurons and Murine Embryonic Fibroblasts (MEFs) extracted from DJ-1 and PON2 deficient mice. The PON2 lactonase activity was assessed to determine the effects of DJ-1 on basal activity and oxidative-induced response of this protein. In addition, in vitro survival assays were performed to examine the role of PON2 in protecting neurons against MPP^+, an *in vitro* model of PD.

DJ-1 knockout mice were generated in the laboratory of Dr. Tak W. Mak, University of Toronto, by Dr. Raymond H. Kim. PON2 deficient mice were generated in Dr. Srinivasa T. Reddy's laboratory, University of California in Los Angeles (UCLA). Mohammad Parsanejad designed all the experiments, and performed most part of them, including PON2 activity assays utilizing DHC. Dr. Noam Bourquard, from UCLA, performed PON2 activity assays utilizing C12 substrate and HPLC system. Mohammad Parsanejad, Yi Zhang, Drs. Hossein Aleyasin, Isabella Ircher and David S. Park wrote the manuscript. Dr. Maxime W.C. Rosseaux maintained DJ-1 transgenic mice. Yi Zhang, Drs. Maxime W.C Rosseaux and Dianbo Qu contributed in generation of molecular constructs and performing the immuno-precipitation assays. Steve Callaghan and Dominique C. Vaillant generated and/or amplified the adenoviruses used in this research project. Dr. Daniel Figeys carried out the mass spectrometry analysis and proteomic screen.

DJ-1 interacts with and regulates Paraoxonase-2, an enzyme critical for neuronal survival in response to oxidative stress

Mohammad Parsanejad[1], Noam Bourquard[2], Yi Zhang[1], Maxime W.C. Rousseaux[1,3], Dianbo Qu[1], Hossein Aleyasin[1], Isabella Irrcher[1,4], Steve Callaghan[1], Dominique C. Vaillant[1], Raymond H. Kim[5] ,Ruth S Slack[1], Tak W. Mak[5], Srinivasa T. Reddy[2], Daniel Figeys[6] and David S. Park[1,7]

[1] Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario, Canada, K1H 8M5

[2] Department of Medicine and Department of Molecular and Medical Pharmacology, David Geffen School of Medicine at UCLA, Los Angeles, California, USA

[3] Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas.

[4] Department of Ophthalmology, Queen's University, Kingston, Ontario

[5] The Campbell Family Institute for Breast Cancer Research, Toronto, Ontario, Canada, M5G 2C1

[6] Ottawa Institute of Systems Biology (OISB), University of Ottawa, Ottawa, Ontario, Canada, K1H 8M5

[7] Department of Cogno-Mechatronics Engineering, Pusan National University, Korea

Running title: Protective role of DJ-1 through Paraoxonase-2

To whom correspondence should be addressed: David S. Park, Ottawa Health Research Institute, Neuroscience Group, University of Ottawa, 451 Smyth Road, Ottawa, Ontario, Canada K1H8M5.

Key Words: Apoptosis/ MPP^+/ Neuron/ Parkinson's disease/PON2

Abbreviation used: PD Parkinson's Disease

ROS Reactive oxygen species

DHC Dihydrocumarin

C12 3-oxo-C12-homoserin lacton

ABSTRACT

Loss-of-function mutations in DJ-1 (PARK7) gene account for about 1% of all familial Parkinson's disease (PD). While its physiological function(s) are not completely clear, DJ-1 protects neurons against oxidative stress in both *in vitro* and *in vivo* models of PD. The molecular mechanism(s) through which DJ-1 alleviates oxidative stress-mediated damage remains elusive. In this study, we identified Paraoxonase2 (PON2) as a direct interacting target of DJ-1. PON2 activity is elevated in response to oxidative stress and DJ-1 is crucial for this response. Importantly, we showed that PON2 deficiency hypersensitizes neurons to oxidative stress induced by MPP^+ (1-methyl-4-phenylpyridinium). Conversely, over-expression of PON2 protects neurons in this death paradigm. Interestingly, PON2 effectively rescues DJ-1 deficiency-mediated hypersensitivity to oxidative stress. Taken together, our data suggest a model by which D-1 exerts its antioxidant activities through regulation of PON2.

INTRODUCTION

PD is a progressive neurodegenerative disorder characterized by selective loss of the pigmented dopaminergic neurons of the Substantia nigra pars compacta (SNc) (Hirsch *et al.* 1988), and reduction in striatal dopamine level. The majority of PD cases do not follow a genetic inheritance pattern (Tanner *et al.* 1999). However, rare familial forms of this disease with their causative genes have been identified (Polymeropoulos *et al.* 1997, Kitada *et al.* 1998, Leroy *et al.* 1998, Kruger *et al.* 1998).

DJ-1 was identified as one of these PD-related genes (Bonifati *et al.* 2003). It was first identified as an oncogene and associated with fertility factors (Nagakubo *et al.* 1997, Klinefelter *et al.* 1997). However, recent evidence in several families showed linkage of homozygous loss of function mutations in DJ-1 to early onset PD (Bonifati et al. 2003, van Duijn *et al.* 2001). The mechanisms by which loss of DJ-1 function promotes PD are unclear. However, it has been most associated with management of reactive oxygen species (ROS). For example, our previous data

demonstrated that DJ-1 null mice are hypersensitive to dopaminergic toxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Kim *et al.* 2005). Consistent with this, numerous reports utilizing *in vitro* and *in vivo* models in both mammalian and drosophila systems support the idea that DJ-1 plays a neuroprotective role under pathological conditions where oxidative stress predominates (Gu *et al.* 2009, Inden *et al.* 2006, Lev *et al.* 2009, Taira *et al.* 2004, Lavara-Culebras & Paricio 2007, Martinat *et al.* 2004, Aleyasin *et al.* 2010, Aleyasin *et al.* 2007). How DJ-1 may regulate ROS is not completely clear. DJ-1 is oxidized on its cysteine residues which are also critical for the ability of DJ-1 to manage ROS (Canet-Aviles *et al.* 2004). DJ-1 also possesses atypical peroxiredoxin activity, although this activity is weak compared to other antioxidant enzymes (Andres-Mateos *et al.* 2007). Others have demonstrated that DJ-1 somehow regulates Nrf2, a master transcription factor for a variety of antioxidant enzymes (Clements *et al.* 2006). However, whether this is true in neurons is controversial (Gan *et al.* 2010).

Recently, to further examine the underlying mechanism(s) by which DJ-1 exerts protection, we performed a proteomics interaction screen for DJ-1 interacting partners. By mass spectrometric analyses, we identified Paraoxonase-2 (PON2) as a novel interacting candidate for DJ-1. PON2 is a member of Paraoxonase family of genes (Paraoxonase-1, 2, 3), which are located as a cluster on chromosome 7 in human and chromosome 6 in mouse. PON2 is ubiquitously expressed in a wide variety of tissues and localized in cytoplasm and membranous structures, such as plasma membrane (Ng *et al.* 2001), endoplasmic reticulum(Horke *et al.* 2008), and mitochondria (Devarajan *et al.* 2011). Several *in vitro* and *in vivo* studies indicate a role for PON2 in diminishing oxidative stress (Ng *et al.* 2006, Ng et al. 2001, Mackness *et al.* 1991). For example, PON2 deficient HeLa cells exhibit elevated intracellular oxidative level which can be reversed by over-expression of PON2 (Ng et al. 2001). PON2 deficiency in mice increases the risk of oxidative stress-related pathophysiological conditions such as development of atherosclerotic lesions (Ng et al. 2006, Devarajan et al. 2011). Furthermore, numerous studies on several human populations reported the association of PON2 polymorphisms with severe ischemic stroke (Lazaros *et al.* 2010), sporadic

amyotrophic lateral sclerosis (SALS) (Slowik *et al.* 2006, Saeed *et al.* 2006, Valdmanis *et al.* 2008) and Alzheimer's disease (AD) (Shi *et al.* 2004, Janka *et al.* 2002). Polymorphisms in another PON member, PON1, have also been associated with susceptibility to PD (Akhmedova *et al.* 2001, Carmine *et al.* 2002). However, the role of PON2 in the context of neuronal loss induced by oxidative stress is unknown.

Given the initial interaction data from our proteomics screen, we examined whether DJ-1 may modulate susceptibility to oxidative stress through regulation of the PON2 enzyme. We provide evidence that DJ-1 interacts with and promotes PON2 activity in the presence of oxidative stress and that this mechanism is one central mechanism by which DJ-1 promotes survival.

MATERIAL AND METHODS

Proteomic screen - The process of the proteomic screen was described before (Ewing *et al.* 2007). Briefly, The data was generated using an LCQ Deca mass spectrometer. Mascot version 1.9 (Matrix Sciences, www.matrixscience.com) was used to analyze the obtained spectra by searching against a human protein sequence database with 122989 entries. This database was generated utilizing the main sources of human protein sequences including GenBank, TrEMBL, SwissProt, IPI, Ensembl. The settings to run the Mascot were as follows: search mode: MS/MS Ion, fixed modification: carbamidomethyl on cysteine, variable modification: oxidation on methionine, peptide mass tolerance: 2Da, fragment mass tolerance: 0.4Da, maximum missed cleavages: 2, enzyme: trypsin. Identification of peptide and protein was performed based on the following criteria: a Mascot peptide score threshold of 15 for single peptide hit proteins.

Cortical neuron culture - Cortical neuron cultures were prepared as described before (Zhang *et al.* 2010, Zhang *et al.* 2006). Briefly, embryos were extracted at 14.5-15.5 days gestation. Their

cortices were dissected and incubated with 0.50 mg/ml trypsin with shaking for 20 minutes at 37°C in Hank's balanced salt solution. Trypsinization was stopped with 0.2 mg/ml trypsin inhibitor and 0.2 mg/ml DNaseI at room temperature. Cells were spun down at 150g and triturated in Neurobasal medium containing 0.2 mg/ml trypsin inhibitor and 0.25 mg/ml DNaseI. Cells were pelleted and resuspended in Neurobasal medium containing B-27 and N-2 supplements and 0.5 mM glutamine. Cells were then plated in dishes pre-coated with poly-D-lysine.

GST pull down assay, Immunoprecipitation (IP) and immunoblotting - Samples (HEK293 cells for IP of over-expressed proteins and primary cortical neurons for IP of endogenous proteins) were washed with phosphate buffered saline (PBS) and harvested and lysed in lysis buffer (50 mM Tris HCl pH7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.2% NP-40 and protease inhibitor). Lysate was cleared of cell debris with centrifugation at 17000xg for 20 minutes and supernatant was used for IP. In the case of GST pull down assay in cells expressing GST-DJ-1, cleared cell lysate was incubated with 20 µl glutathione sepharose for 2-4 hours. In other cases, cell lysate was incubated with 2-4 µg of antibodies overnight and with TrueBlot IgG beads for 2 hours. Precipitated complexes were washed 3 times with lysis buffer and eluted by boiling in 2x SDS-loading buffer. Proteins were separated on 10% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. The membrane was blocked with 1% milk for 1 hour at room temperature and treated with primary antibody overnight to probe the target protein. Membrane was washed 3 times and treated with TrueBlot secondary antibody (to avoid IgG signal) for 1 hour.

Membrane extraction and Paraoxonase2 activity using 3-oxo-C12-homoserine lacton - Cells were homogenized in homogenization buffer (5 mM Tris/HCl pH 7.4, 1 mM $CaCl_2$ and EDTA-free protease inhibitor). Homogenized cells were pelleted at 17000xg for 30 minutes, resuspended in extraction buffer (25 mM Tris/HCl pH 7.4, 1 mM $CaCl_2$, 10% glycerol, 1% w/v dodecyl-β-d-maltoside (DDM) and EDTA-free protease inhibitor) and incubated at 4°C with agitation overnight

for complete resuspension. Cell debris was extracted with centrifuging at 2000 xg for 5min. For PON2 activity, 4 µg of crude membrane extracts prepared from cultured cortical neurons or MEFs was incubated with 10 µM 3-oxo-C12-homoserine lactone (C12) in a 50 µl volume of 25 mM Tris-HCl, pH 7.4, and 1 mM $CaCl_2$ at room temperature. Reactions were stopped with an equal volume of acetonitrile, and 5 µl was used to measure C12 by quantitative autoinducer bioassay using E.coli MG4 containing pKDT17 (Clements et al. 2006). The P. aeruginosa lasB gene is activated with 3-oxo-C12-homoserine lacton (C12). E.coli MG4 containing a plasmid with *lasB::lacZ* transcriptional fusion (pKDT17), can be induced by C12 to activate Beta-galactosidase gene. Beta-galactosidase will then hydrolyze ortho-Nitrophenyl-β-galactoside (ONPG) to ortho-nitrophenol with yellow color. The more C12 remaining in the buffer, the more signal will be produced by beta-galactosidase activity. For this assay, E.coli MG4 (pKDT17) was divided to 1 ml aliquots. 0.01 ml of membrane samples (already treated with C12) was added to each aliquot and incubated for 4 hours at 37°C. 0.1 ml of the culture was added to 1 ml of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM beta-mercaptoethanol) and vortexed for 10 seconds. 0.1 ml of the mixture was transferred to a 96 well plate in triplicates and Z buffer only was used as blank. 0.02 ml of ONPG was added to each well and incubated for 10 minutes at room temperature. Reaction was stopped with 0.05 ml of 1 M Na_2CO_3 and ONPG signal was read at 420 nm. (Pearson *et al.* 1994, Pearson *et al.* 1995, Cooley *et al.* 2010, Gray *et al.* 1994)

PON2 activity using Dihydrocumarin as a substrate - Intact cells were washed with PBS and incubated with activity buffer (50 mM Tris-HCl pH7.4, 1 mM CaCl2 and 1 mM Dihydrocumarin (DHC) as substrate) at room temperature. UV absorbance at 270 nm was measured after 10 minutes incubation. One unit of PON2 activity is equal to 1 µmol DHC hydrolyzed/ml/min (Shiner *et al.* 2007).

In vitro Adenoviral gene delivery, MPP^+ treatment and survival assessment - Adenoviral infection was performed at a multiplicity of infection (MOI) of 30 at the time of plating. For survival assays, 48 hours after plating, the cultures were treated with 20 µM of MPP^+ for 48 hours as previously described (Qu *et al.* 2007, Huang *et al.* 2010). Cultures were then fixed with 4% Paraformaldehyde (PFA), washed 2 times with PBS and stained with Hoechst 33258 (0.5 ng/ml). The percentage of surviving neurons was calculated as the number of GFP-positive neurons with intact nucleus over the total number of GFP-positive neurons (Aleyasin *et al.* 2004). For survival assays with no adenoviral infection, primary cortical neurons obtained from PON2 deficient or wild type mice were subjected to 10, 20 and 40 µM MPP^+ treatment for 48 hours. Cells were lysed and the survival rate was assessed by direct microscopy and counting intact nuclei.

Statistical analysis - Statistical significance was assessed by Anova on data obtained from three independent experiments. All data are presented as mean ± SEM, and significance is marked by * in case of $p<0.05$, ** in case of $p< 0.01$ and *** in case of $p<0.001$.

RESULTS

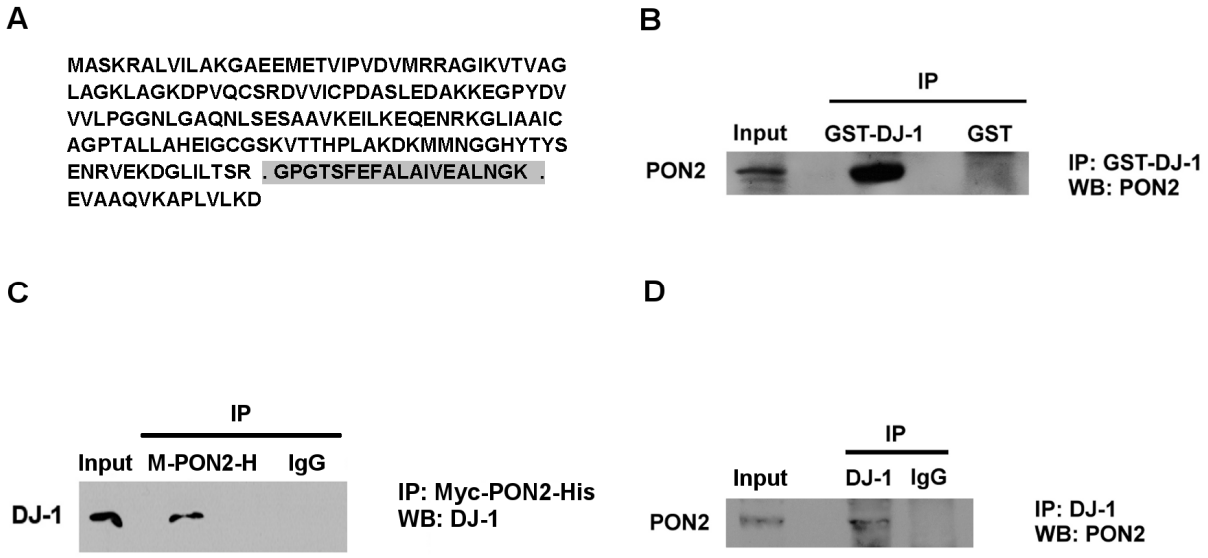
DJ-1 interacts with PON2

We previously reported a systems biological approach to generation of a large scale human protein-protein interaction map as a valuable tool for understanding proteins functions and the mechanisms of disease (Ewing *et al.* 2007). This map was generated based upon a screen utilizing a large number of human bait proteins (407 unique bait proteins) mostly known for their role in diseases such as breast cancer, colon cancer, diabetes and obesity. These bait proteins were used to immunoprecipitate potential interacting partners subsequently identified through mass spectrometric analyses.

Our original data set was filtered with a number of criteria designed to eliminate false positive and non specific interactions which eliminated a large number of valid potential interactors. These

exclusion criteria included targets which appeared to interact with more than 5% of bait proteins. Accordingly, we reanalyzed our data sets with focus on DJ-1 eliminating these exclusion criteria. We further analyzed select DJ-1 interacting candidates with proper biochemical interaction studies to further validate any potential hits obtained through our systems biology directed screen.

In this study we report the identification and characterization of a new DJ-1 interacting partner, Paraoxonase-2 (PON2). We initially identified DJ-1 through peptide analyses using PON2 as bait (mascot score 30.2, Figure 2.1A). We next confirmed the interaction of DJ-1 and PON2 in HEK293 cells. The initial experiments were performed utilizing expressed DJ-1. Plasmids expressing GST-DJ-1 were transfected into HEK293 cells and analyses performed by affinity precipitating with glutathione sepharose beads and Western blot analyses for endogenous PON2, utilizing a PON2 antibody. In figure 2.1B, we show that expression of GST-DJ-1 but not a GST control plasmid immunoprecipitates PON2. The reciprocal experiment was also performed, HEK293 cells were transfected with a vector expressing Myc-PON2-His. PON2 was immunoprecipitated with a Myc antibody and immunoblotted for endogenous DJ-1 utilizing a DJ-1 antibody (Figure 2.1C). In figure 2.1C, we show that immunoprecipitation with Myc antibody but not an IgG control antibody reveals interaction with DJ-1. Finally, we tested whether both endogenous PON2 and D-1 interact in neurons. We carried out co-immunoprecipitation-western blot assay using cultured murine cortical neurons. Endogenous DJ-1 was immunoprecipitated with DJ-1 antibody and immunoblotted with PON2 antibody. As shown in figure 2.1D, PON2 was co-immunoprecipitated with DJ-1 antibody but not IgG control antibody. Taken together, this indicates that PON2 associates with DJ-1 *in vivo*.


A
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AGPTALLAHEIGCGSKVTTHPLAKDKMMNGGHYTYS
ENRVEKDGLILTSR . GPGTSFEFALAIVEALNGK .
EVAAQVKAPLVLKD
B
IP
Input
GST-DJ-1
GST
PON2
IP: GST-DJ-1
WB: PON2
C
IP
Input
M-PON2-H
IgG
DJ-1
IP: Myc-PON2-His
WB: DJ-1
D
IP
Input
DJ-1
IgG
PON2
IP: DJ-1
WB: PON2

Figure 2.1

Figure 2.1. DJ-1 and PON2 interact. (**A**) DJ-1 full length protein sequence. Peptide observed from DJ-1 after using PON2 as bait is highlighted. Mascot peptide score is 30.2. (**B**) HEK293 cells expressing GST-DJ-1 or GST as control were lysed and GST-DJ-1 was precipitated by glutathione sepharose beads and analyzed with western blotting using PON2 antibody. (**C**) HEK293 cells were transfected with plasmid expressing Myc-PON2-His (M-PON2-H). Cells were lysed and Myc-PON2-His was precipitated with Myc antibody. Isolated complexes were analysed with western blotting using DJ-1 antibody. (**D**) DJ-1 was pulled down by DJ-1 antibody from cell lyate extracted from cultured cortical neurons. Immune complexes were analysed with western blotting using PON2 antibody.

Effects of DJ-1 and oxidative stress on PON2 activity

Previous reports have shown that PON2 lactonase activity increases in response to oxidative stress (Rosenblat *et al.* 2003). Given that DJ-1 interacts with PON2, we hypothesized that DJ-1 modulates PON2 activity in this paradigm. To test this hypothesis, we measured PON2 lactonase activity in cortical neurons derived from DJ-1 wild-type (WT) or knockout (KO) embryos treated with MPP^+ (20 μM), for 12 hours. MPP^+ is a complex I inhibitor which leads to oxidative stress and death of a number of different neurons (Desai *et al.* 1996, Ramsay & Singer 1986, Ramsay *et al.* 1987, Poirier & Barbeau 1985, Sinha *et al.* 1986). PON2 lactonase activity was first measured by assessing the percentage of hydrolysis of PON2 specific substrate, 3-oxo-C12-homoserine lactone (C12) by PON2 (Ng et al. 2006). As shown in figure 2.2A, PON2 activity is significantly elevated after MPP^+ treatment in wild-type neurons. Remarkably DJ-1 deficiency not only blocked PON2 basal lactonase activity, but also blocked MPP^+-induced enzymatic activity. We then confirmed this result using a second assay protocol which involves hydrolysis of dihydrocumarin (DHC), a lactone which can be hydrolyzed by PON2 (Rosenblat et al. 2003, Shiner et al. 2007, Shiner *et al.* 2004). Similarly, with this assay, oxidative stress induced PON2 activity only in WT neurons and not in DJ-1 deficient neurons (Figure 2.2B). To further confirm this observation, we measured hydrolysis of DHC in another cell type challenged with a different oxidative reagent. Indeed, PON2 activity was also elevated in response to oxidative stress induced by hydrogen peroxide (100 μM for 24 hours) in WT mouse embryonic fibroblasts (MEFs) but not in DJ-1 KO MEFs (Figure 2.2C). This supports the idea that DJ-1 regulates PON2 activity in multiple cellular contexts and ROS conditions. Finally, we determined whether low PON2 activity observed under conditions of DJ-1 deficiency could be rescued by DJ-1 expression. Accordingly, we expressed DJ-1 or GFP in DJ-1 WT or KO MEFs (Figure 2.2D). DJ-1 KO MEFs expressing GFP have less PON2 activity measured by C12. This activity in DJ-1 KO MEFS expressing DJ-1 increases by almost 59%. Taken together, these results indicate that loss of DJ-1 impairs PON2 activity and that this loss can be rescued by DJ-1 re-expression.

Importantly, we also determined the effects of DJ-1 expression in PON2 deficient (PON2 def) cells. We expressed DJ-1 or GFP as control in PON2 WT or deficient MEFs and PON2 activity was measured as described above. As shown in figure 2.3A, DJ-1 expression in PON2 WT MEFs induced PON2 activity by almost 51% compared to GFP control group. However, the induced activity observed in PON2 deficient MEFs was dramatically lower (less than 5%). These results indicate that the lactonase activity induced by DJ-1 is almost exclusively through PON2.

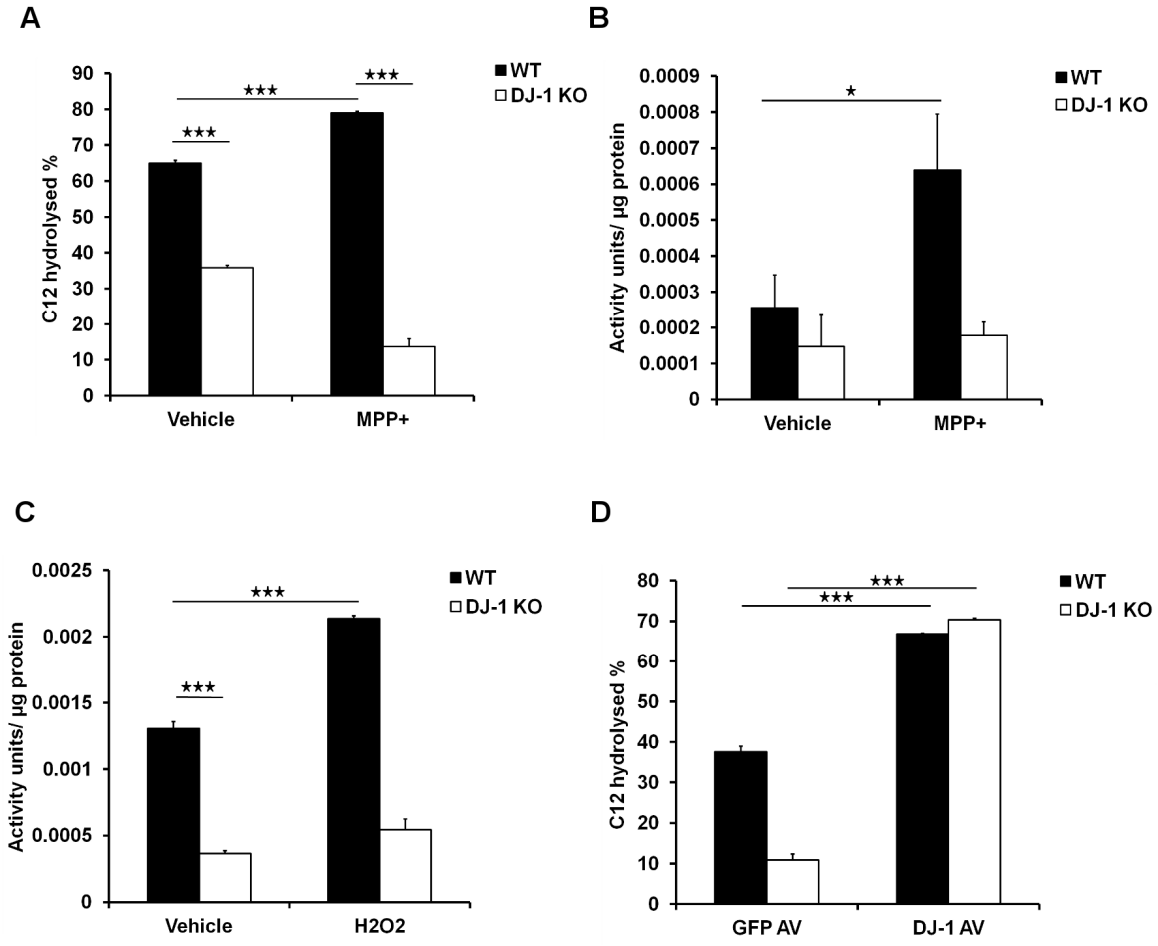
A
B
C
D
WT
DJ-1 KO
C12 hydrolysed %
Activity units/ µg protein
Vehicle
MPP+
H2O2
GFP AV
DJ-1 AV
***
*

Figure 2.2

Figure 2.2. DJ-1 and oxidative stress modulate PON2 activity. (**A**) Cultured WT and DJ-1 KO cortical neurons were treated with MPP^+ (20 μM) for 12 hours and cells were washed and membrane was extracted. Crude membrane was exposed to the substrate C12 for 60 minutes and the percentage of remaining C12 was measured. (**B**) Cultured WT and DJ-1 KO cortical neurons were treated with MPP^+ (20 μM) for 24 hours. Neurons were then exposed to DHC for 10 minutes and the amount of hydrolysis of DHC was assessed with measuring UV absorbance. One unit of PON2 activity is equal to 1 μmol DHC hydrolyzed/ml/min. (**C**) WT and DJ-1 KO MEFs were treated with hydrogen peroxide (100 μM) for 24 hours and PON2 activity was measured as described in B. (**D**) WT and DJ-1 KO MEFs were infected with adenovirus expressing DJ-1 or GFP alone as control. After 48 hours of expression, cells were lysed and exposed to C12 as the substrate for 60 minutes. Percentage of C12 remaining in activity buffer was measured. * denotes $p< 0.05$, ** denotes $p<0.01$, and *** denotes $p<0.001$, Anova.

DJ-1 does not affect PON2 protein level in neurons

DJ-1 is reported to interact with RNA and/or localize to the nucleus (van der Brug *et al.* 2008, Hod *et al.* 1999). Accordingly, it is possible that DJ-1 acts through regulation of transcription/translation/stabilization of PON2 and that direct interaction demonstrated above, is not necessary for the modulation of PON2 by DJ-1. To examine this possibility, we treated cortical neurons obtained from DJ-1 WT or KO embryos with MPP^+ (20 μM) for 0, 6, 12 and 24 hours and compared their PON2 protein levels using western blot analysis. Our data demonstrates that there is no significant difference in PON2 protein level between DJ-1 WT and KO neurons. In addition, PON2 protein level does not change in response to MPP^+ induced oxidative stress (Figure 2.3B). This observation rules out the possibility that DJ-1 increases PON2 activity through increasing PON2 protein levels.

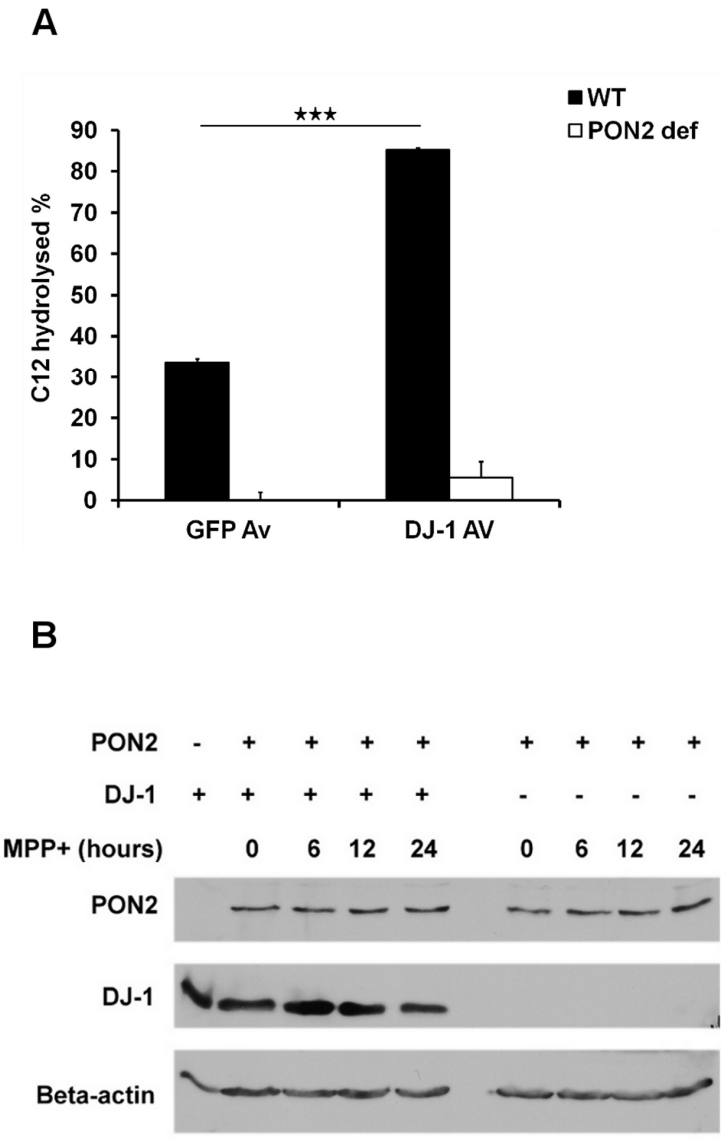
A
WT
PON2 def
***
C12 hydrolysed %
90
80
70
60
50
40
30
20
10
0
GFP Av
DJ-1 AV
B
PON2 - + + + + + + + +
DJ-1 + + + + + - - - -
MPP+ (hours) 0 6 12 24 0 6 12 24
PON2
DJ-1
Beta-actin

Figure 2.3

Figure 2.3. DJ-1 has no lactonase activity and no effects on PON2 protein level. (**A**) WT and PON2 deficient MEFs were infected with adenovirus expressing DJ-1 or GFP. PON2 activity was then measured using C12 as described before. (**B)** Cultured cortical neurons extracted from DJ-1 WT and DJ-1 KO were treated with MPP^+ (20 μM) for different durations. Cells were lysed and PON2 protein level was assessed by western blotting. * denotes $p< 0.05$, **denotes $p<0.01$ and *** denotes $p<0.001$, Anova.

PON2 protects against MPP^+ induced neuronal death

Loss of DJ-1 results in hypersensitization to a number of death-inducing oxidative stress stimuli. If the regulation of PON2 by DJ-1 is biologically significant we would anticipate that a) PON2 loss would also sensitize neurons to oxidative stress and b) PON2 expression would rescue the sensitization to stress induced by loss of DJ-1. This would also suggest PON2 as a downstream target of DJ-1. To test this hypothesis, we first treated PON2 WT or deficient cortical neurons with 0, 10, 20 and 40 μM MPP^+ for 48 hours and assessed the neuronal cell survival by nuclear integrity. Our data shows that PON2 deficient neurons are significantly hypersensitive to MPP^+ treatment when compared to neurons from WT littermate controls (Figure 2.4A). To confirm the protective function of PON2, we expressed PON2 or GFP alone as control in WT or PON2 def cortical neurons. The cells were exposed to 20 μM MPP^+ for 48 hours and their survival was assessed by counting proportion of GFP positive cells with intact nuclei to total GFP positive cells, as described previously (Kim et al. 2005). Our data demonstrate that PON2 expression rescues PON2 deficiency-mediated hypersensitivity to MPP^+ (Figure 2.4B). Finally, we examined whether PON2 expression can also rescue DJ-1 loss-mediated hypersensitivity to MPP^+. To test this, we expressed GFP-PON2 or GFP alone as control by adenoviral infection in DJ-1 WT or KO cortical neurons. After treatment with MPP^+ (20 μM) for 48 hours, the cell survival was assessed as above. Consistent with our hypothesis, PON2 expression protects neurons against MPP^+ and can also reverse the hypersensitivity observed with DJ-1 loss (Figure 2.4C).

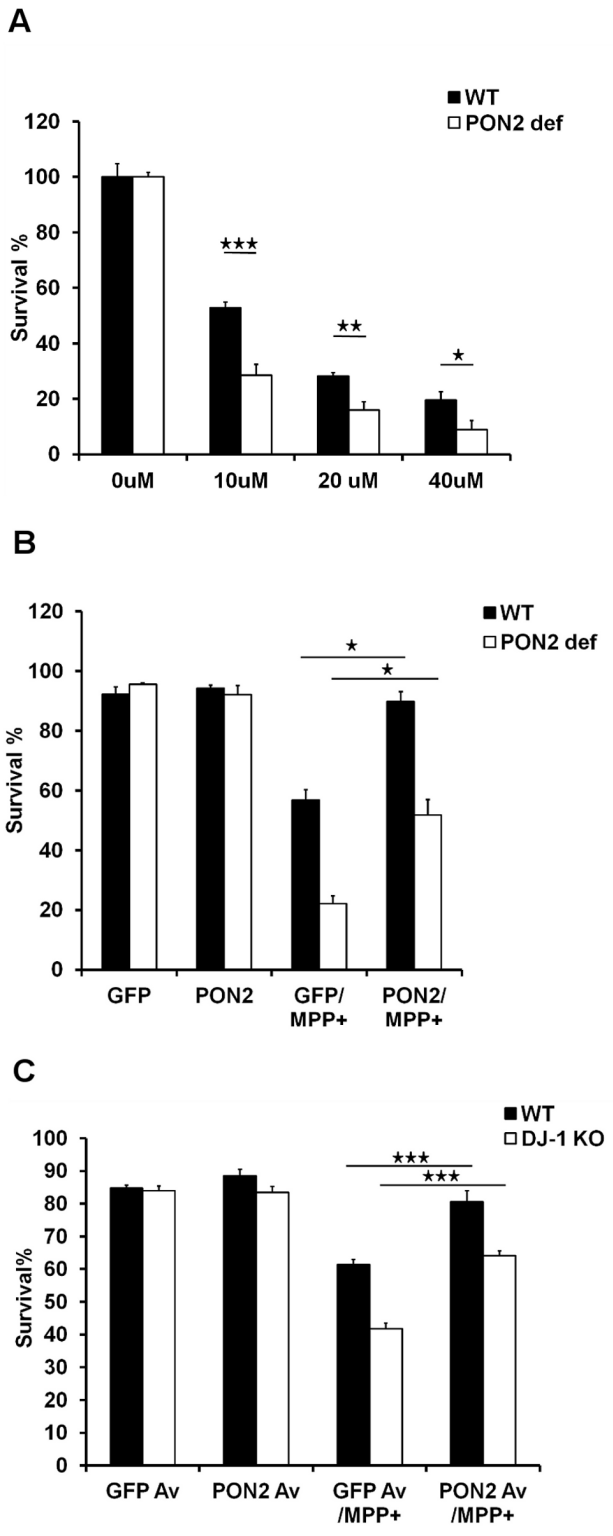
A
WT
PON2 def
Survival %
120
100
80
60
40
20
0
***
**
*
0uM
10uM
20 uM
40uM
B
WT
PON2 def
Survival %
120
100
80
60
40
20
0
*
*
GFP
PON2
GFP/ MPP+
PON2/ MPP+
C
WT
DJ-1 KO
Survival%
100
90
80
70
60
50
40
30
20
10
0
***
***
GFP Av
PON2 Av
GFP Av /MPP+
PON2 Av /MPP+

Figure 2.4

Figure 2.4. PON2 protects neurons against MPP^+. (**A**) Primary cortical neurons obtained from PON2 deficient or wild type mice were subjected to 10, 20 and 40 μM MPP^+ treatment for 48 hours. Cells were lysed and viability was assessed by direct microscopy and counting intact nuclei. (**B**) WT and PON2 def cortical neurons were transfected with PON2 or GFP-expressing plasmids and subjected to 20 μM MPP^+ for 48 hours. Cells were fixed and the nuclei were stained with Hoechst. Survival percentage represents the ratio of GFP-expressing cells with morphologically intact nuclei to the total number of GFP positive cells. (**C**) WT and DJ-1 KO cortical neurons over-expressing PON2-GFP and GFP alone as control (using adenovirus expressing PON2 or GFP) were subjected to 20 μM MPP^+ for 48 hours. The survival assay was performed as described in part B. * denotes $p< 0.05$, **denotes $p<0.01$ and *** denotes $p<0.001$, Anova.

DISCUSSION

Several studies have demonstrated the link between DJ-1 and oxidative damage in neurodegeneration (Aleyasin et al. 2007, Kim et al. 2005, Aleyasin et al. 2010, Gu et al. 2009, Inden et al. 2006). The purpose of the present study was to investigate the mechanism(s) underlying the capacity of DJ-1 to mediate survival. In an initial mass spectrometry screen for DJ-1 interacting protein, we identified PON2 as a candidate interacting partner. We confirmed this interaction, particularly under endogenous conditions in primary neurons. The model by which DJ-1 is a critical factor in regulating PON2 activity is supported by several observations. First, elevated PON2 activity which occurs in response to MPP^+ mediated oxidative stress is dependent upon DJ-1. Multiple cells types including neurons and MEFs have lowered PON2 activity in the absence of DJ-1 in response to oxidative stress. This deficiency can be rescued by DJ-1 expression. Importantly, our results also suggest that manner by which DJ-1 regulates PON2 is not through more potentially indirect effects on PON2 stability since DJ-1 deficiency has no effect on PON2 levels. Instead we suggest a model by which DJ-1 may more directly modulate PON2 activity based upon the evidence presented for direct physical interaction between the two proteins. However, this must be confirmed by additional studies which rely on first identifying the interaction domains between DJ-1 and PON2.

Second, we show that PON2 itself is critical for regulating survival in response to conditions of oxidative stress (in particular induced by MPP^+). Neurons deficient in PON2 are more sensitive to MPP^+ treatment which can be rescued by re-introduction of PON2. These results are consistent with the notion that PON2 is known to lower ROS (Ng et al. 2001, Ng *et al.* 2005, Ng et al. 2006, Mackness et al. 1991). Interestingly, DJ-1 deficient neurons are also similarly hypersensitive to oxidative stress and this hypersensitivity can be reversed by PON2 expression. This observation is consistent with the model by which DJ-1 acts to increase the activity of PON2. Note, that while these observations imply that DJ-1 is a critical regulator of PON2, it is not an absolute requirement for PON2 activity.

The mechanism by which PON2 lactonase activity relates to reduced oxidative stress is unclear. One possibility is that the lactonase activity per se is essential for regulation of death and oxidative stress. Multiple lines of evidence have shown that environmental factors such as pesticide exposure can increase the risk of early onset of Parkinson's disease (Godeiro *et al.* 2010, Tanner *et al.* 2011, Sanyal *et al.* 2010). Paraoxon is an organophosphorus compounds, active metabolite of the insecticide parathion, whose toxicity is due to their strong anticholinesterase action. Evidence has shown that paraoxon can cause apoptotic cell death in proliferating cells through activation of mitochondrial pathways (Saleh *et al.* 2003). Paraoxon-induced AChE inhibition can aggravate experimental Parkinsonism triggered by MPTP in mice (Ben-Shaul *et al.* 2006), suggesting paraoxonase may play a role in defending against Parkinson etiologic factors. Therefore, in this scenario, the defined lactonase activity of PON2 may somehow indirectly lead to reduced oxidative stress, at least under certain conditions. A second possibility is that the lactonase activity is somehow separate from the oxidative capacity of PON2. In support of this hypothesis, it was reported that the antioxidant capacity could be dissociated from the lactonase activity (Altenhofer *et al.* 2010). It is interesting to speculate that perhaps PON2 might modify the antioxidant capacity of DJ-1 directly. However, our studies indicate that expression of PON2 by itself in the absence of DJ-1 is protective, suggesting that this is not the case. Resolution of these questions will be of critical importance in future studies.

A final interesting point is that while both PON2 and DJ-1 have been localized to numerous subcellular compartments, both have been associated with mitochondrial functions. For example, DJ-1 accumulates in mitochondria (presumably outer mitochondrial membrane) in response to oxidant stress (Junn *et al.* 2009, Canet-Aviles et al. 2004). DJ-1 may also be present in more interior mitochondrial compartments (Zhang *et al.* 2005). The role of DJ-1 in mitochondrial functions has not been fully understood although it has been suggested to be essential for the survival promoting capacity of DJ-1. Similarly PON2 has also been reported in the mitochondria where it binds to coenzyme Q10 (Devarajan et al. 2011). In this regard, it has been shown that PON2 deficient mice

have less complex I and III activity and less ATP production and also elevated mitochondrial ROS generation (Devarajan et al. 2011). It is therefore interesting to speculate that perhaps DJ-1 may interact with PON2 in the mitochondria to regulate antioxidant stress responses. This is an exciting possibility given the increasing association of mitochondrial defects with the mechanisms underlying PD and the number of PD linked genes including DJ-1 associated with mitochondrial quality control (Irrcher *et al.* 2010, Canet-Aviles et al. 2004, Junn et al. 2009). In support of this, we have shown that DJ-1 loss leads to increased ROS production from isolated mitochondria (Irrcher et al. 2010). Whether this relates to the function of DJ-1 on PON2 will also be of interest in future studies.

In summary, we demonstrate that DJ-1, a Parkinson's disease related gene, interacts with PON2 in neurons and cell lines. This interaction appear to modulate PON2 activity as DJ-1 KO cells have less basal PON2 activity and do not respond to oxidative stress as DJ-1 WT cells do. This effect can be reversed by expression of DJ-1. In addition, expression of PON2 in DJ-1 KO neurons is more protective against Parkinson's model of neuronal death than expression of DJ-1 in PON2 deficient background.

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The authors declare no conflict of interest.

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CHAPTER 3

Regulation of the VHL/HIF-1 pathway by DJ-1

Statement of authors contribution:

Our work presented in this manuscript investigates the role of DJ-1 in regulation of VHL ubiquitination activity, and also the potential effects of downstream target of VHL, HIF-1, in protecting neurons against MPP^+ model of neuronal loss.

Mohammad Parsanejad designed and carried out the experiments with assistance from Yi Zhang, Maxime W.C Rousseaux and Hossein Aleyasin. Dianbo Qu contributed in immuno-precipitation experiments and generated molecular constructs used in ubiquitination assay. Isabella Irrcher provided the human PD samples. Steve Callaghan made the adenoviral vector used in survival experiments. Dr. Stephen Lee contributed reagents and scientific advice. Dr. Daniel Figeys performed initial proteomic screen. The manuscript was written by Mohammad Parsanejad, Yi Zhang and Dr. David Park with assistance from Drs. Ruth S. Slack and Tak W. Mak.

Regulation of the VHL/HIF-1 pathway by DJ-1

Mohammad Parsanejad[a], Yi Zhang[a], Dianbo Qu[a], Isabella Irrcher[a,b], Maxime W.C. Rousseaux[a,1], Hossein Aleyasin[a], Fatemeh Kamkar[a], Steve Callaghan[a], Ruth S. Slack[a], Tak W. Mak[c], Stephen Lee[a] ,Daniel Figeys[d] and David S. Park[a,e]

[a] Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, ON, Canada, K1H 8M5

[b] Department of Ophthalmology, Queen's University, Kingston, Ontario, Canada, K7L 5G2

[c] The Campbell Family Institute for Breast Cancer Research, Toronto, Ontario, Canada, M5G 2C1

[d] Institute of Systems Biology (OISB), University of Ottawa, Ottawa, Ontario, Canada, K1H 8M5

[e] Department of Cogno-Mechatronics Engineering, Pusan National University, Korea

[1] Present address: Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas.

Running title: Regulation of the VHL/HIF-1 Pathway by DJ-1

Correspondence to: David S. Park, Ottawa Health Research Institute, Neuroscience Group, University of Ottawa, 451 Smyth Road, Ottawa, Ontario, Canada K1H8M5.

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ABSTRACT

DJ-1 (PARK7) is a gene linked to autosomal recessive Parkinson disease (PD). We showed previously that DJ-1 loss sensitizes neurons in models of PD and stroke. However, the biochemical mechanisms underlying this protective role are not completely clear. Here, we identify Von Hippel Lindau (VHL) protein as a critical DJ-1-interacting protein. We provide evidence that DJ-1 negatively regulates VHL ubiquitination activity of HIF-1α by inhibiting HIF/VHL interaction. Consistent with this observation, DJ-1 deficiency leads to lowered HIF-1α levels in models of both hypoxia and oxidative stress, two stresses known to stabilize HIF-1α. We also demonstrate that HIF-1α accumulation rescues DJ-1 deficient neurons against 1-methyl-4-phenylpyridinium (MPP^+) induced toxicity. Interestingly, lymphoblast cells extracted from DJ-1-related PD patients show impaired HIF-1α stabilization when compared to normal individuals, indicating that the DJ-1/VHL link may also be relevant to a human context. Taken together, our findings delineate a model by which DJ-1 mediates neuronal survival by regulation of the VHL/ HIF-1α pathway.

INTRODUCTION

PD is a neurodegenerative disorder, which is pathologically characterized by progressive loss of dopaminergic (DA) neurons in the *substantia nigra pars compacta* (SNc). While most cases are sporadic, 5-10% of the cases are familial and monogenic. Loss of DJ-1 leads to an early onset form of PD (Bonifati et al., 2003). We have previously shown that DA neurons in DJ-1-deficient mice exhibit hypersensitivity to oxidative stress, induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), H_2O_2 and MPP^+, the active metabolite of MPTP (Kim et al., 2005). We and others have also shown that reactive oxygen

species (ROS) is increased in cells/mice lacking DJ-1 (Taira et al., 2004; Aleyasin et al., 2007). In line with this observation, numerous studies demonstrated that DJ-1 is neuroprotective under oxidative conditions (Aleyasin et al., 2007; Lee et al., 2009; Aleyasin et al., 2010). The mechanisms through which DJ-1 exerts its antioxidant-neuroprotective role are largely unclear.

To identify potential DJ-1-interacting proteins, we performed an unbiased mass spectrometry screen, which indicated Von Hippel Lindau (VHL) protein as a potential interacting partner. VHL is primarily a tumor suppressor protein whose mutations lead to a rare autosomal dominant inherited disease which presents as tumors in several tissues (Lonser et al., 2003). The best-known biochemical role of VHL is E3 ubiquitin-ligase, in complex with Cul-2, elongin B and C (Lonergan et al., 1998). One of the best defined substrates of VHL is the α-subunit of hypoxia-inducible factor-1 (HIF-1α), a transcription factor important in the hypoxic response (Kamura et al., 2000; Tanimoto et al., 2000). Under normal levels of oxygen or ROS, proline residues of HIF-1α are hydroxylated by prolyl-hydroxylase proteins (PHD) (Berra et al., 2003), ubiquitinated by VHL protein and finally degraded by the proteasome. Hypoxia or increased levels of ROS, however, inhibit PHD, which, in turn, inhibits VHL-mediated degradation of HIF-1α and induces transcription of genes involved in cellular adaptation to hypoxic and oxidative stress condition such as vascular endothelial growth factor (VEGF) and erythropoietin (EPO). Interestingly, HIF-1α expression and its downstream targets are down-regulated in the SNc of PD brains (Elstner et al., 2011). Conversely, increase of HIF-1α and/or its targets are protective in several models of PD (Genc et al., 2002; Lee et al., 2009). HIF-1α also up-regulates tyrosine hydroxylase and increases dopamine release in PC12 cells and rat ventral mesencephalic

cells (Johansen et al., 2010). Given these links of HIF-1α to PD, and our observations that VHL could potentially interact with DJ-1, we explored whether DJ-1 could regulate the VHL/HIF pathway. Presently, we provide evidence to support a model by which DJ-1 regulates the ability of VHL to interact with and ubiquitinate HIF-1α with consequences to neuronal survival in an *in vitro* model of PD.

MATHERIAL AND METHODS

Immunoprecipitation (IP)

RCC cells were infected with adenovirus expressing FLAG-VHL-GFP and lysed in lysis buffer (50 mM Tris HCl pH7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.2% NP-40 and protease inhibitor). The lysate (cleared of cell debris) was incubated with FLAG antibody overnight. Protein-antibody complexes were pulled down by Protein A sepharose beads and FLAG-VHL-GFP was eluted from FLAG antibody with 10ug 3X FLAG peptides. Proteins were then analyzed by western blot using both VHL and DJ-1 antibodies. In the second set of experiments RCC cells were transfected with pXJ40 plasmid expressing GST-DJ-1 or GST as control. GST-DJ-1 was purified with glutathione sepharose beads. Beads were then washed 3 times with lysis buffer and boiled in 4X SDS-sample buffer. Protein complexes were analyzed by western blot, using DJ-1 and VHL antibodies.

Semi quantitative reverse transcription-PCR

Cortical neurons, from primary culture, were harvested in Trizol and their total RNA was extracted based on Invitrogen protocols and 60 ng of extracted RNA was used for each reaction. Reactions were performed utilizing SuperScript One-Step RT-PCR kit with Platinum *Taq* (Invitrogen). The programs designed for RT-PCRs were as follows: cDNA

synthesis, 48°C for 45 min, initial denaturation, 94°C for 2 min, amplification of the target genes, 28 (HIF-1α and VEGF) or 26 (S12) cycles of 94°C for 30 s, 59°C (HIF-1α) or 60°C (VEGF and S12) for 30 s, and 72°C for 1 min. Primers used for amplification of the target genes were as follows: HIF-1α, 5′-ctaacaagccgggggaggac -3′ and 5′-tgtggctgggagttcttcgtatta-3′ to generate a 476bp product, VEGF, 5′-gacttgtgttgggaggagga-3′ and 5′-cgtgtttctggaagtgagca-3′ to produce a 161bp product, S12, 5′-ggaaggcatagctgctgg-3′ and 5′-cctcgatgacatccttgg-3′. After resolving the RT-PCR products on a 1.5% agarose–ethidium bromide gel, the signal density of the obtained bands were analyzed by densitometry. The signal density of the target bands were normalized against those of S12 and then control values (untreated control for each experiment) and were reported as fold increase in signal density of the target gene in reference to untreated control from the same experiment. Data are presented as mean ± SEM (n=3).

In vitro Ubiquitination assay

To assess VHL ubiquitination activity, *in vitro* ubiquitination assay was performed as previously described (Kamura et al., 2000), with minor modifications. Briefly, ≈ 5µg aliquots of purified FLAG-VHL-GFP complex (purified as described above) were incubated with ≈0.3µg Uba1, ≈0.6µg hUbc5a, ≈50µg ubiquitin, ≈0.5µg GST-HIF-1α and ≈0.5µg purified His-DJ-1 in a 40µl reaction buffer (40 mM Hepes-NaOH pH 7.9, 60 mM potassium acetate, 2 mM DTT, 5 mM $MgCl_2$, 0.5 mM EDTA pH 7.9, 10% glycerol, and 2 mM ATP) at 30°C for 2 hours. GST-HIF-1α was immunoprecipitated from the reaction mixture by glutathione sepharose and equal amounts of protein complexes were subjected to SDS-PAGE analysis. To assess its ubiquitination and VHL-interaction level, the western blot membrane was probed with ubiquitin and VHL antibody respectively.

Hypoxia, MPP^+ treatment and survival assessment

72 hours after plating cortical neurons or MEFs, half of the media was removed and the dishes ,with no lid on, were transferred to hypoxia chamber (1% O_2). For survival experiments, cortical neurons were treated with 1mM DMOG for 2 hours. Cells were then incubated with 20 μM MPP^+ for 48 hours (Qu et al., 2007; Huang et al., 2010) and lysed in lysis buffer (10× PBS, 10% Triton X-100, 1 m $MgCl_2$, and 5% cetyldimethylethylammonium bromide) and their survival was assessed by counting morphologically intact nuclei (Galehdar et al., 2010). For survival assay involving adenoviral infection (SHIF-1α adenovirus), cells were infected with SHIF-1α or GFP-expressing adenovirus. After 48 hours, they were treated with 20 μM MPP^+ for 48 hours, fixed with 4% paraformaldehyde and their nuclei were stained with Hoechst 33258 (0.25 μg/ml) to assess nuclear morphology. The survival percentage represents the ratio of GFP-expressing cells with morphologically intact nucleus to the total number of GFP-expressing neurons (Aleyasin et al., 2004).

Statistical analysis

Statistically significant differences were determined with ANOVA and post hoc Tukey's test or student T-test (when the comparison was between two groups). Data were shown as mean ± SEM, and significance was denoted by * where $p<0.05$ and by ** where $p<0.01$ and *** where $p<0.001$.

RESULTS

Identification of VHL as a DJ-1-interacting protein

To identify upstream regulators or downstream effectors for DJ-1 that might be involved in the intracellular signalling pathways in disease models, we searched for cellular proteins that are associated with DJ-1 using a mass spectrometric strategy. This screen was previously described on detail (Ewing et al., 2007). Analysis of the dataset suggested VHL protein as a potential DJ-1 interacting candidate with the interaction confidence score of 0.31.

We confirmed the physical interaction of DJ-1 and VHL proteins by expressing GST-DJ-1 (Figure 3.1A) in Renal Cell Carcinoma (RCC) cells. GST-DJ-1 was immunoprecipitated with glutathione sepharose beads and protein complexes were analysed using VHL antibody. GST-DJ-1 (Figure 3.1A) shows physical binding with endogenous VHL. This interaction was further confirmed in reciprocal experiments where FLAG-VHL-GFP (F-VHL-G) was expressed in RCC cells and following FLAG immunoprecipitation, probed for endogenous DJ-1 (Figure 3.1B). We observed that endogenous DJ-1 was co-immunoprecipitated with VHL.

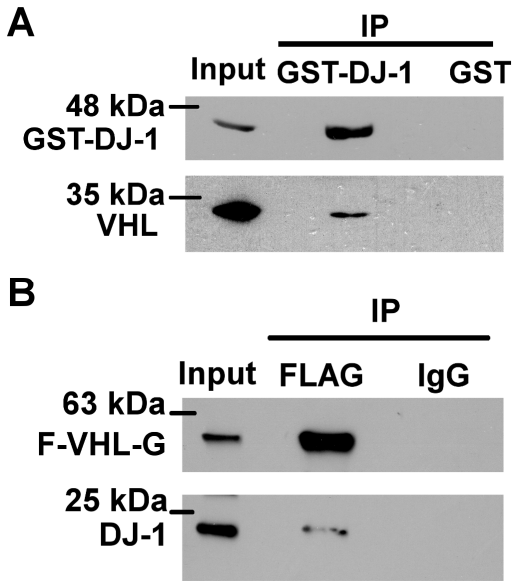
A
IP
Input
GST-DJ-1
GST
48 kDa
GST-DJ-1
35 kDa
VHL
B
IP
Input
FLAG
IgG
63 kDa
F-VHL-G
25 kDa
DJ-1

Figure 3.1

Figure 3.1. DJ-1 interacts with VHL: (A) GST-DJ-1 or GST as control was over-expressed in Renal Cell Carcinoma cells (RCC cells) and immunoprecipitated by glutathione sepharose beads. Precipitated proteins were then subjected to western blot analysis using VHL antibody. (B) RCC cells expressing FLAG-VHL-GFP (F-VHL-G) were lysed and FLAG-VHL-GFP was immunoprecipitated with FLAG antibody and subjected to SDS-PAGE analysis using DJ-1 antibody.

DJ-1 regulates HIF-1α stability

To explore the functional consequences of this interaction, we examined the potential regulatory role of DJ-1 on VHL activity. It is well established that VHL increases the proteolysis of HIF-1α through a ubiquitination-dependent process (Maxwell et al., 1999; Kamura et al., 2000). Thus, we examined HIF-1α levels, as an indicator of VHL activity, in response to hypoxia and oxidative stress in both DJ-1 deficient murine neurons and embryonic fibroblasts (MEFs). Wide-type (WT) and knockout (KO) cells were incubated under hypoxic (1% oxygen) or normoxic conditions. As figure 3.2A shows, HIF-1α levels are decreased in hypoxia treated DJ-1 KO cortical neurons compared to WT controls. Similar results were also observed using MEFs (Figure 3.2B). WT and DJ-1 KO cells were also exposed to different doses of H_2O_2, and assessed for HIF-1α. These results also show that DJ-1 modulates HIF-1α stability in response to oxidative stress (Figure 3.2C and 2D).

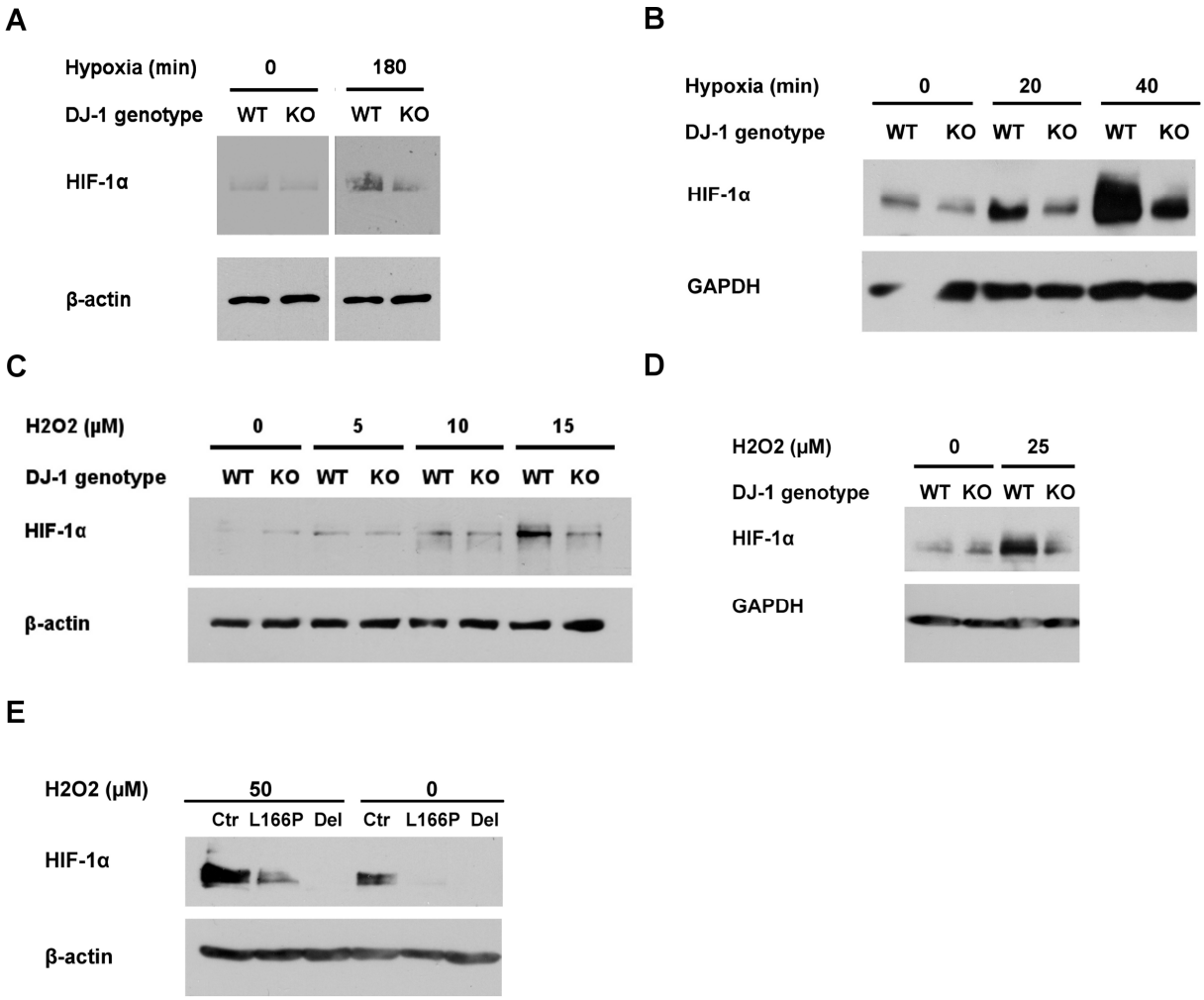
A
Hypoxia (min)
0
180
DJ-1 genotype
WT KO WT KO
HIF-1α
β-actin
B
Hypoxia (min)
0
20
40
DJ-1 genotype
WT KO WT KO WT KO
HIF-1α
GAPDH
C
H2O2 (μM)
0
5
10
15
DJ-1 genotype
WT KO WT KO WT KO WT KO
HIF-1α
β-actin
D
H2O2 (μM)
0
25
DJ-1 genotype
WT KO WT KO
HIF-1α
GAPDH
E
H2O2 (μM)
50
0
Ctr L166P Del Ctr L166P Del
HIF-1α
β-actin

Figure 3.2

Figure 3.2. Functional link between DJ-1 and VHL: Wild type or DJ-1 KO (A) cortical neurons or (B) MEFs were incubated in normal condition or in hypoxia (1% oxygen) for 180 min (neurons) or 20 and 40 minutes (MEFs). (C) Cortical neurons were treated with 0, 5, 10 and 15 μM H_2O_2 for 2 hours. (D) Wild type and DJ-1KO MEFs were also exposed to 0 or 25 μM H_2O_2 for 24 hours. Cells were lysed and HIF-1α protein level was assessed with western blot using HIF-1α antibody. (E) Lymphoblasts extracted from two Parkinson's cases, one with a deletion in DJ-1 (Del) and one with a point mutation in DJ-1 (L166P), or from control (Ctr) individual were exposed to 50 μM hydrogen peroxide for 24 hours and their HIF-1α response was examined with western blot analysis using HIF-1α antibody.

HIF-1α response is VHL dependent and controlled at post-translational level

The difference observed with the HIF-1α response in DJ-1 WT and DJ-1 KO cells could be VHL independent and controlled at the transcriptional level. In order to address this, we first assessed the HIF-1α mRNA levels, utilizing reverse transcriptase PCR (RT-PCR) analysis, following oxidative or hypoxic conditions in DJ-1 WT and KO cortical neurons. As figures 3.3A and B show, there is no change in HIF-1α mRNA levels in either DJ-1 WT or KO neurons in response to hypoxia or oxidative stress. These results were confirmed with quantitative real time RT-PCR analyses (data not shown).

We further explored the role of DJ-1 on HIF-1α-regulated gene expression by assessing whether the downstream HIF-1 target gene, VEGF, is altered with DJ-1 deficiency. Consistent with lower HIF-1α levels, the induction of VEGF mRNA was reduced with DJ-1 loss (Figure 3.3A and B).

If differences in HIF-1α protein levels with DJ-1 deficiency are VHL dependent, we would anticipate that either inhibition of the proteasome or inhibition of prolyl hydroxyase activity would bypass VHL-dependent regulation of HIF-1α. HIF-1α levels, then, should be similar in both DJ-1 WT and KO cells. We tested this, utilizing the proteasome inhibitor MG-132 and prolyl hydroxylase inhibitor dimethyloxallyl glycine (DMOG). Following these treatments, we could detect no gross difference in total HIF-1α content between DJ-1 WT and KO neurons (Figure 3.3C). Taken together, these results indicate that differences observed in the HIF-1α levels in the presence or absence of DJ-1 is mostly through VHL-dependent proteasomal degradation.

DJ-1 negatively regulates VHL ubiquitin ligase activity *in vitro*

We next asked whether DJ-1 may directly regulate VHL-mediated ubiquitination activity. To investigate this possibility, we performed a VHL *in vitro* ubiquitination assay in

the presence or absence of DJ-1. To isolate active VHL complex, RCC cells were infected with adenovirus expressing FLAG-VHL-GFP and then VHL was immunoprecipated using FLAG antibody. Recombinant hUBA1 and hUBC5a served as ubiquitin-activating enzyme E1 and ubiquitin-conjugating enzyme E2, respectively. Finally, bacterially expressed recombinant human HIF-1α (GST-HIF-1α), hydroxylated by VHL deficient RCC cell (786-0) lysate, was used as substrate. All the above proteins were incubated with ubiquitin and ATP (Kamura et al., 2000) in presence or absence of bacterially expressed human DJ-1, for 2 hours. GST-HIF-1α was then precipitated with glutathione sepharose beads and analyzed by western blotting with ubiquitin and VHL antibodies. As shown in figure 3.3D, the presence of DJ-1 decreases the level of HIF -1α ubiquitination. In addition, our data indicate that the presence of DJ-1 is associated with a weaker VHL-HIF-1α interaction (Figure 3.3D), consistent with the model that DJ-1 leads to reduced HIF-1α ubiquitination. Taken together, these results suggest that DJ-1 reduces HIF-1α ubiquitination by reducing interaction with VHL.

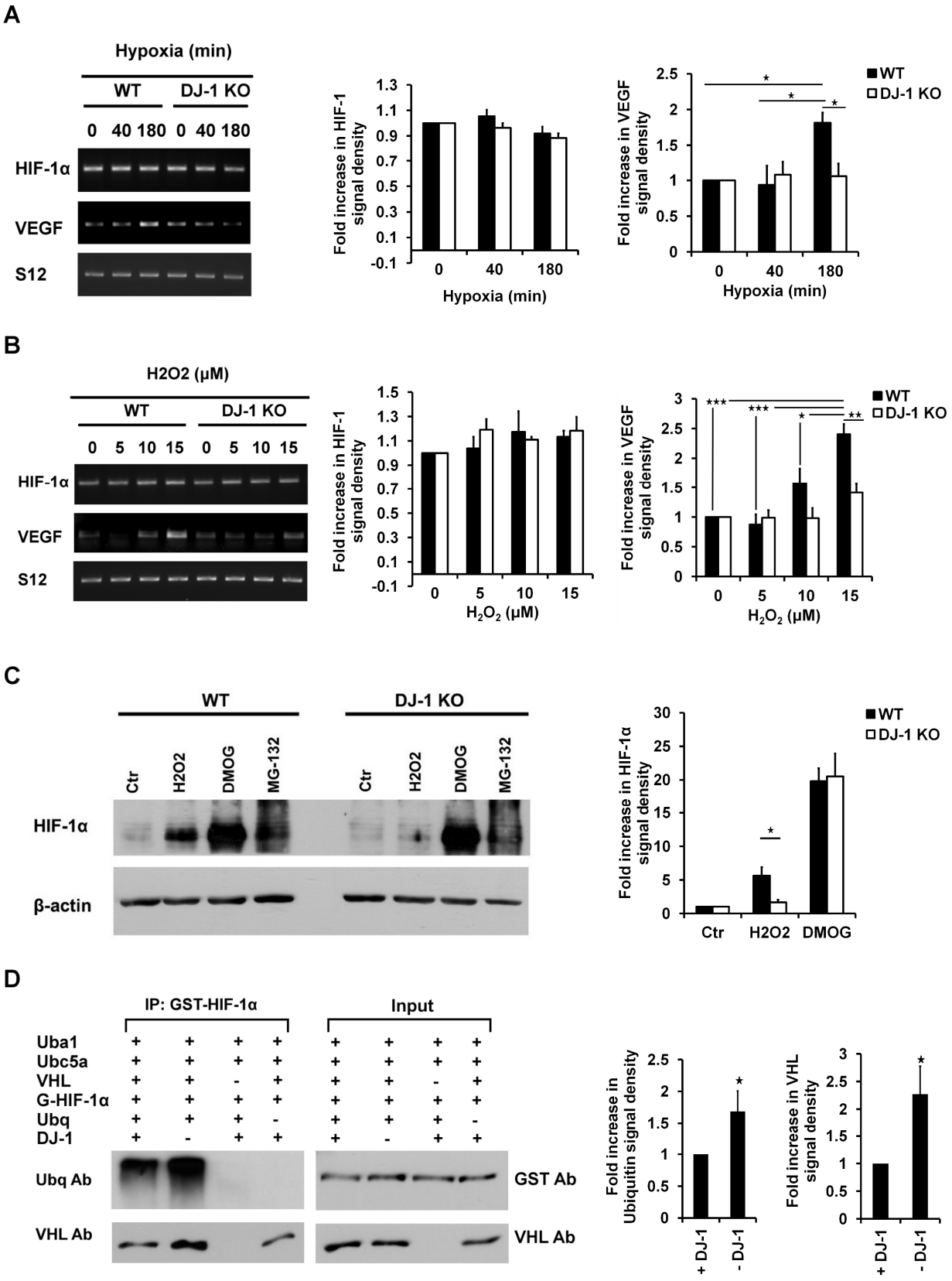
A
Hypoxia (min)
WT
DJ-1 KO
0 40 180 0 40 180
HIF-1α
VEGF
S12
Fold increase in HIF-1 signal density
Hypoxia (min)
Fold increase in VEGF signal density
WT
DJ-1 KO
B
H2O2 (μM)
WT
DJ-1 KO
0 5 10 15 0 5 10 15
HIF-1α
VEGF
S12
Fold increase in HIF-1 signal density
H2O2 (μM)
Fold increase in VEGF signal density
WT
DJ-1 KO
C
WT
DJ-1 KO
Ctr
H2O2
DMOG
MG-132
HIF-1α
β-actin
Fold increase in HIF-1α signal density
WT
DJ-1 KO
Ctr
H2O2
DMOG
D
IP: GST-HIF-1α
Input
Uba1
Ubc5a
VHL
G-HIF-1α
Ubq
DJ-1
Ubq Ab
VHL Ab
GST Ab
VHL Ab
Fold increase in Ubiquitin signal density
+ DJ-1
- DJ-1
Fold increase in VHL signal density
+ DJ-1
- DJ-1

Figure 3.3

Figure 3.3. HIF-1α expression is equal in DJ-1 WT and KO neurons and its response is VHL dependent: (A) WT or DJ-1 KO cortical neurons were exposed to hypoxia for 40 and 180 minutes or (B) to different concentrations of H_2O_2 (0, 5, 10 and 15 μM) for 2 hours. HIF-1α transcripts were amplified by RT-PCR. The densitometry analysis of each experiment is demonstrated in the middle and right panels. (C) WT and DJ-1 KO cortical neurons were treated with 15μM H_2O_2 for 2 hours, 1mM DMOG for 3 hours and 20μM MG-132 for 3 hours and HIF-1α level was assessed by western blot using HIF-1α antibody. The densitometry analysis is demonstrated in the right panel with the exception of MG-132 treatment, which was difficult to quantify due to the smearing inherent with ubiquitination. (D) *In vitro* VHL ubiquitination assay was performed by incubating GST-HIF-1α protein with the components of its ubiquitination pathway including hUBA1, hUBC5A, VHL complex, in presence or absence of DJ-1 protein, at 30ºC for 2 hours. Groups without VHL or ubiquitin were used as controls. After reaction, GST-HIF-1α (G-HIF-1α) was precipitated from the assay mixture with glutathione sepharose beads, and its ubiquitination level and ability to interact with VHL were assessed by western blot analysis using ubiquitin and VHL antibodies respectively. Total content of VHL and GST-HIF-1α in each reaction, before starting ubiquitination is shown as input. Densitometry analysis of ubiquitination and VHL-HIF-1 interaction is shown in the middle and right panels. Each bar is the mean ± SEM from three independent experiments. * denotes $p< 0.05$, ** denotes $p<0.01$, and *** denotes $p< 0.001$ ANOVA or student T-test.

HIF-1 rescues DJ-1 deficient neurons in an *in vitro* model of PD

DJ-1 deficient neurons have been reported to be hypersensitive to oxidative stress induced by several agents including MPP^+ (Kim et al., 2005). If HIF-1 is a downstream target of DJ-1 in this death paradigm, we predicted that HIF-1 stabilization could rescue DJ-1 deficiency-mediated hypersensitivity to MPP^+. We first tested this hypothesis using DMOG to induce HIF-1α stabilization. Cultured DJ-1 WT or KO cortical neurons were pretreated with DMOG and then treated with MPP^+ (20 μM) for 48 hours, lysed in lysis buffer and the morphologically intact nuclei were counted. As figure 3.4A shows, DMOG-mediated HIF-1 accumulation rescues DJ-1 deficient neurons against MPP^+. We also investigated the protective effect of stable HIF-1α (SHIF-1α) expression. SHIF-1α contains point mutations that substitutes alanine instead of proline residues in the oxygen degradable domain (P402A/ P564A) leading to resistance to hydroxylation by PHD (Smith et al., 2005). As demonstrated in figure 3.4B, adenovrial mediated expression of GFP-SHIF-1α also rescues DJ-1 KO neurons against oxidative stress induced by MPP^+. Figure 3.4C demonstrates the expression of GFP-SHIF-1α in cultured cortical neurons. Adenoviral-mediated expression of GFP-SHIF-1α was also examined by western blot in RCC cells infected with this virus (Figure 3.4D).

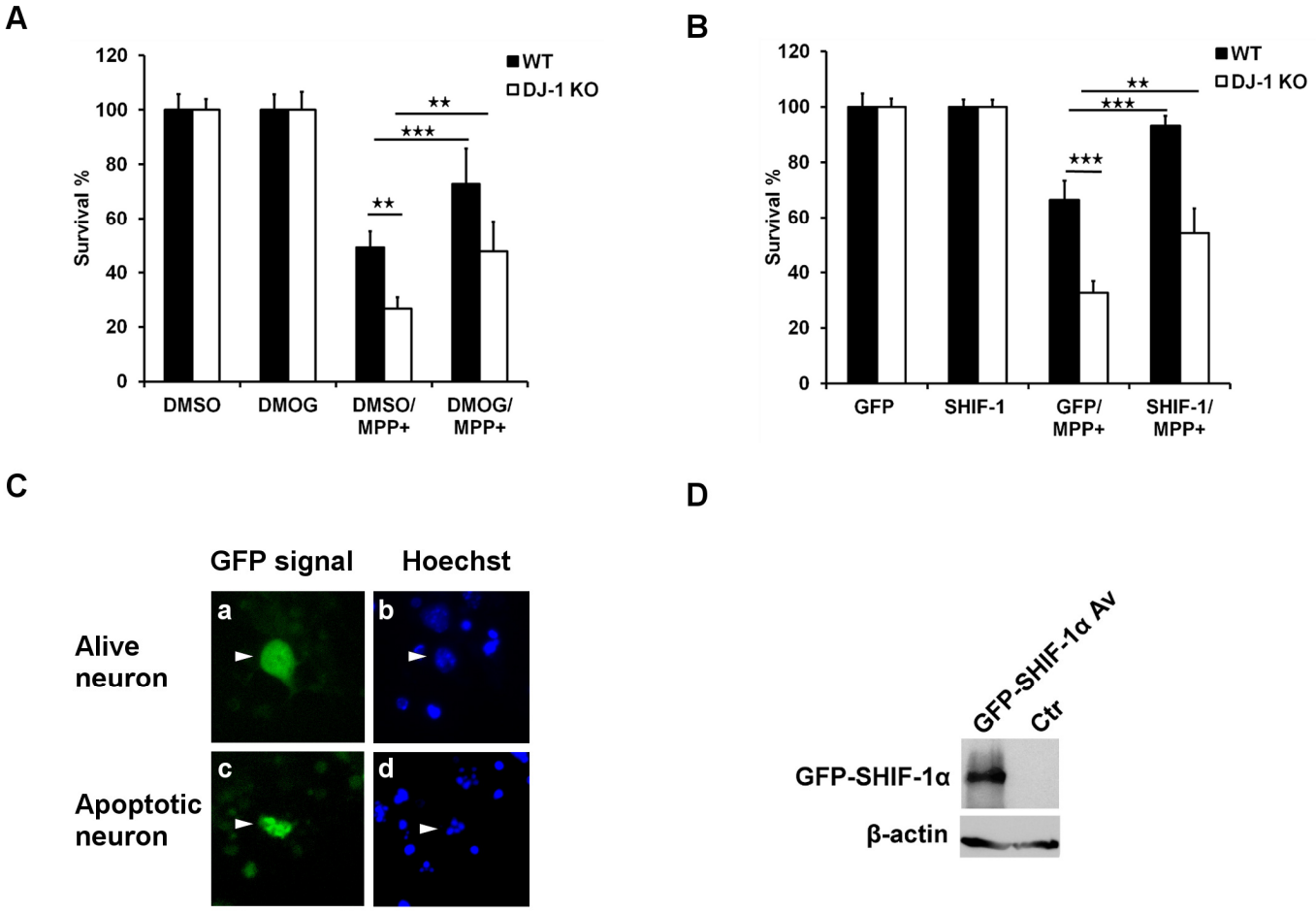
A
120
100
80
60
40
20
0
Survival %
WT
DJ-1 KO
**
***
**
DMSO
DMOG
DMSO/ MPP+
DMOG/ MPP+
B
120
100
80
60
40
20
0
Survival %
WT
DJ-1 KO
**
***
***
GFP
SHIF-1
GFP/ MPP+
SHIF-1/ MPP+
C
GFP signal
Hoechst
Alive neuron
Apoptotic neuron
a
b
c
d
D
GFP-SHIF-1α Av
Ctr
GFP-SHIF-1α
β-actin

Figure 3.4

Figure 3.4. HIF-1 over-expression protects neurons against MPP^+: (A) Primary cortical neurons derived from DJ-1 KO or WT mouse embryos were pre-treated with DMOG (1mM) for two hours and then treated with MPP^+ (20 μM) for 48 hours. Neurons were lysed and the intact nuclei were counted. (B) Stable form of HIF-1α (GFP-SHIF-1α) or GFP alone as control was over-expressed in WT and DJ-1 KO cortical neurons by GFP-SHIF-1α and GFP-expressing adenovirus. Cells were then exposed to 20 μM MPP^+ for 48 hours and their fixed nuclei were stained with Hoechst. Their viability was examined with calculating the ratio of neurons with intact nuclei and GFP signal (C, a and b) to the total number of GFP-expressing cells. (C) Adenoviral expression of GFP-SHIF-1α in cultured cortical neurons (a and c) and Hoechst staining to detect intact versus apoptotic nuclei (b and d). (D) Detection of GFP-SHIF-1α by western blot, using HIF-1α antibody, in RCC cells infected with GFP-SHIF-1α-expressing adenovirus. Each bar is the mean ± SEM of three independent experiments. * denotes $p< 0.05$, ** denotes $p<0.01$, and *** denotes $p< 0.001$ ANOVA test.

HIF-1α stabilization in response to oxidative stress is reduced in DJ-1 mutated Parkinson's patients

To better relate our findings to human PD condition, we determined whether HIF-1α protein levels differed in lymphoblasts cultured from DJ-1-related PD cases (Irrcher et al., 2010). The PD patients included two individuals with different mutations in DJ-1: one with a point mutation, replacing leucine 166 with proline (L166P) and the other with a deletion mutation from exon 1 to 5 (Del). The extracted lymphoblasts were treated with 50 μM H_2O_2 or vehicle as control for 24 hours and then the HIF-1α accumulation was assessed by western blot. As Figure 3.2E shows, oxidative stress-induced HIF-1α accumulation is significantly diminished in DJ-1-linked PD lymphoblast samples compared to control patients (Figure 3.2E). These results confirm that the defective stress response of HIF-1α observed in DJ-1 WT and KO cells also occurs in DJ-1-associated PD cases.

DISCUSSION

DJ-1 has been reported as a neuroprotective protein under oxidative and hypoxic conditions (Kim et al., 2005; Aleyasin et al., 2007; Aleyasin et al., 2010). The mechanisms through which DJ-1 manages oxidative damage are unclear. In a proteomic screen via a mass spectrometry analysis, we identified VHL protein as a DJ-1 interactor. The interaction of DJ-1 and VHL was further confirmed in immunoprecipitation studies. In conjunction with previous reports indicating a protective role of both DJ-1 and the VHL target protein HIF-1α, we hypothesized that DJ-1 protects neurons against oxidative damage via inhibition of VHL-ubiquitination activity. Our present observations support this hypothesis. First, HIF-1α levels in rodent cells (MEFS and neurons) under oxidative and/or hypoxic conditions are lower with DJ-1 deficiency. This is not due to difference in transcription of HIF-1α. Second,

in a direct assay of VHL ubiquitination activity utilizing isolated components, we show that DJ-1 reduces ubiquitination of HIF-1α. Third, stabilization of HIF-1α reversed the sensitivity to MPP^+-induced neuronal death, observed with DJ-1 deficiency. Finally, we demonstrate that our observations in rodent cells also have relevance to human cells extracted from DJ-1-related PD patients, where HIF-1α response to oxidative stress is less compared to control samples. Taken together, our results support a model by which regulation of VHL activity by DJ-1 mediates its survival properties.

The significance of our results is supported by the notion that the VHL and HIF-1α have been implicated in a number of degenerative models. For example HIF-1α signaling has been shown to be down-regulated in SNc of PD cases (Elstner et al., 2011). In addition, accumulation of HIF-1α or its downstream proteins such as EPO and VEGF results in reduction of MPTP toxicity in mice (Genc et al., 2002; Lee et al., 2009). They also have neuroprotective effects in ischemia (Sun et al., 2003; Kilic et al., 2005). Given the notion that DJ-1 deficiency sensitizes to neuronal damage in both toxin and stroke models of injury, our results link these observations by providing a mechanism through which DJ-1 regulates the VHL/HIF pathway to promote survival.

Interestingly, DJ-1 has been reported to interact with a number of E3 Ligases, such as Parkin, PIASxα, TOPORS and TRAF6 (Takahashi et al., 2001; Shinbo et al., 2006; Xiong et al., 2009; Zucchelli et al., 2010). This suggests a common theme of action for DJ-1, although the functional significance of these interactions has never been clarified. It is therefore significant that we demonstrate the functional outcome of such interaction in the case of VHL. This novel action of DJ-1 may work in conjunction with other D-1 functions previously described. This includes its ability to interact with RNA (van der Brug et al., 2008), Daxx (Junn et al., 2005), PSF (Zhong et al., 2006), Parkin E3 ubiquitin ligase

complex (Xiong et al., 2009), and AKT pathway (Aleyasin et al., 2010). How/whether these functions are related remain to be seen. Interestingly, some of these signals may interact. For example, the AKT pathway has been shown to modulate HIF-1α response (Vasseur et al., 2009) and we have shown that DJ-1 modulates AKT signaling (Aleyasin et al., 2010). In this way DJ-1 may coordinate multiple signals including VHL to coordinate survival.

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CHAPTER 4

General discussion

Parkinson's disease (PD) is the second most prevalent neurodegenerative disease. The complex nature of PD has made it difficult to completely understand the mechanisms underlying this disease. Several pathogenic processes such as oxidative stress, unfolded protein stress, inflammation and mitochondrial dysfunction have been proposed as common events contributing to PD progression. However, whether/how these events mediate the initial degenerative process is unclear.

One significant advancement in understanding the cause of PD came from finding that approximately 10% of all PD cases show familial inheritance. The identification of genes, that when mutated cause familial PD, has given rise to new investigative tools in hopes of revealing the molecular mechanisms underlying sporadic PD pathogenesis. DJ-1 is one such gene whose loss-of-function mutations cause an autosomal recessive form of PD. Numerous studies, including work done in our laboratory, support the protective role of DJ-1 under oxidative conditions (Kim et al., 2005a; Inden et al., 2006; Aleyasin et al., 2007; Gu et al., 2009; Aleyasin et al., 2010). The mechanism(s) of function of this gene is the subject of this PhD dissertation. To this end, using a proteomics screen, we identified Paraoxonase-2 (PON2) and Von Hipple Lindau (VHL) as potential interactors of DJ-1. Interestingly, similar to DJ-1, PON2 and VHL target protein HIF-1 have been repeatedly shown to be protective against oxidative insults. The first part of our study, presented in Chapter Two, provides evidence that DJ-1 enhances PON2 activity and its response to oxidative stress, and that PON2 plays a significant role in protection against neuronal death in an *in vitro* model of dopaminergic cell loss. The second part of our work, presented in Chapter Three, demonstrates that DJ-1 inhibits VHL-mediated ubiquitination of HIF-1α by decreasing the interaction of VHL and HIF-1α, resulting in stabilization of HIF-1α. We also show that HIF-

1 is important in protecting neurons in an *in vitro* model of neuronal death.

The mechanism of function of DJ-1: PON2

In the first part of this thesis, we demonstrated an interaction between DJ-1 and PON2 in primary cortical neurons. We hypothesized that DJ-1 provides protection against oxidative stress by inducing PON2 activity. In support of this hypothesis we have made the following observations. First, oxidative stress-mediated increase of PON2 activity, induced by MPP^+ or H_2O_2, is decreased in DJ-1 deficient cells. This decreased activity of PON2 during oxidative stress is reversed by re-introduction of DJ-1. The reduction in PON2 activity observed with loss of DJ-1 is not mediated through a reduction in PON2 protein level, as PON2 protein level does not change with induction of oxidative stress, nor in the absence of DJ-1. This observation, together with our data showing that DJ-1 and PON2 physically interact, leads us to propose that DJ-1 has a positive modulatory effect on PON2 activity.

Second, PON2 deficiency, like DJ-1 deficiency, results in hypersensitivity of neurons to MPP^+, consistent with previous reports indicating an antioxidant role for PON2 (Mackness et al., 1991; Ng et al., 2001; Ng et al., 2006b). Importantly, hypersensitivity of PON2 deficient neurons can be rescued by re-introduction of PON2. In regards to how DJ-1 and PON2 act together to protect against oxidative stress, we propose three possible pathways: 1) that PON2 acts upstream of DJ-1; 2) that DJ-1 acts upstream of PON2; or 3) that PON2 acts in parallel to DJ-1. We show that over-expression of PON2 in DJ-1 deficient background is protective, ruling out the first proposed pathway. Given our previously-mentioned data, reduced lactonase activity in absence of DJ-1, we predict that DJ-1 is upstream of PON2 and induces the activity of this protein. Whether or how the lactonase activity of PON2 relates to

its ability to reduce oxidative stress remains elusive. One hypothesis is that the lactonase activity is a critical factor for reduction of ROS and/or regulation of pro-survival pathways. One well-characterized group of substrates of PON2 is homoserine lactons, important pro-inflammation agents, which are hydrolyzed by the lactonase activity of PON2 (Juhas et al., 2005; Stoltz et al., 2007; Kim et al., 2011). Given that extensive inflammatory responses contribute to oxidative stress production and are implicated in the cause of PD (McGeer et al., 1988; McGeer et al., 2003), we speculate that the lactonase function of PON2 could be protective via its anti-inflammatory role. This hypothesis is supported by reports that suggest that the lactonase function of PON2 increases in a compensatory mechanism to reduce excessive oxidative stress (Rosenblat et al., 2003; Shiner et al., 2004; Fuhrman et al., 2008). Additionally, this response is oxidative stress-dependent, as reduction of superoxide anion formation by NADPH-oxidase reduces PON2 lactonase activity (Shiner et al., 2004). An alternative hypothesis is that the lactonase activity is independent of the antioxidant function of PON2. This hypothesis is supported by an interesting study, in which a number of point mutations in PON2 independently abolish its lactonase activity but maintain its antioxidant function (Altenhofer et al., 2010). It would be interesting to examine whether lactonase-dead mutants of PON2 could still rescue the hypersensitivity of DJ-1 deficient neurons in the MPP^+ model. If these mutants could not rescue the cell death phenotype, we would suggest that regulation of lactonase activity by DJ-1 is essential for protective role of DJ-1. Conversely, if the phenotype was rescued we would further investigate the role of DJ-1 in regulating antioxidant function of PON2 independently of its lactonase function.

The mechanism(s) underlying the antioxidant function of PON2 is also elusive. A recent study has suggested that PON2 exerts this function through an interaction with dopamine D2

receptor in renal cells and subsequent reduction of NADPH oxidase activity (Yang et al., 2012). Interestingly, DJ-1 is also involved in reduction of NADPH oxidase activity by dopamine D2 receptor (Cuevas et al., 2012). Accordingly, there is a possibility that these proteins work in a complex, where the dopamine D2 receptor may play a scaffolding role to localize DJ-1 and PON2 in close proximity. DJ-1 would then be able to induce PON2 activity, leading to reduction of NADPH oxidase activity and ROS production.

A final and potentially pertinent observation is the sub-cellular localization of PON2 and DJ-1 to mitochondria. Interestingly, both have been implicated in mitochondrial functions (Canet-Aviles et al., 2004; Zhang et al., 2005a; Junn et al., 2009; Devarajan et al., 2011). We have shown that DJ-1 plays a role in maintaining mitochondrial health and dynamics (Irrcher et al., 2010). PON2 is also essential for normal function of complex I and III, ATP production and mitochondrial ROS formation (Devarajan et al., 2011). Accordingly, one possibility of PON2 and DJ-1 action is that DJ-1 enhances PON2 antioxidant and pro-survival functions at mitochondria. This suggested pathway may be of critical importance since mitochondria are major sources of ROS, whose uncontrolled production leads to oxidative damage to almost all components of cells. In addition, given that the normal function of mitochondria is essential for neuronal survival, and mitochondria are one of the central cellular components involved in PD pathogenesis, the role of DJ-1 and PON2 together in maintaining mitochondrial dynamics and controlling ROS production could be an interesting topic to further study.

The mechanism of function of DJ-1: VHL

Our studies indicate that DJ-1 also plays a critical role in regulating VHL function and ultimately downstream signals such as HIF-1, which may play a critical role in the degenerative process of PD. In line with this hypothesis, we have noted the following observations. First, DJ-1 physically interacts with VHL. As mentioned before, VHL is an E3 ligase and interestingly, to this end, several E3 ligases including Parkin (Xiong et al., 2009), TOPORS (Shinbo et al., 2005), PIASxα (Takahashi et al., 2001; Shinbo et al., 2006), and TRAF6 (Zucchelli et al., 2010) have also been identified as interactors of DJ-1. These findings suggest that DJ-1 has a general affinity to E3 ligases, which may be a common mechanism to exert its protective action. The significance of these interactions, particularly in the context of PD, has not been revealed. Thus, our work with clarifying the biochemical and functional effects of this type of interaction is of essential importance.

Second, in mouse cortical neurons or fibroblasts (MEFs) lacking DJ-1, response of HIF-1α and its downstream target, VEGF, to oxidative and hypoxic insult is reduced. As DJ-1 is an mRNA-interacting protein and can also directly or indirectly regulate the expression of several genes (van der Brug et al., 2008; Blackinton et al., 2009b), there is a possibility that this reduction in HIF-1α response occurs at transcriptional/ translational levels, independent of VHL. Our data, however, shows that there is no difference in HIF-1α mRNA level between wild type and DJ-1 deficient neurons. Additionally, after inhibition of prolyl hydroxylase to bypass VHL-mediated degradation of HIF-1α, or inhibition of the proteasome to stabilize the total produced HIF-1α, no differences in HIF-1α protein levels were detected in WT versus DJ-1 null cells. These observations suggest that the effect of DJ-1 on HIF-1α level is a post-translational and VHL-dependent event.

Third, with an *in vitro* ubiquitination assay using purified components of the HIF-1α ubiquitination pathway, we demonstrated that in the presence of DJ-1, both ubiquitination of HIF-1α and interaction between HIF-1α and VHL decrease. This experiment suggests that DJ-1 may physically block binding of VHL to HIF-1α, thus preventing degradation. This type of mechanism of action has previously been reported for DJ-1. For instance, interaction of DJ-1 with PIASx-alpha retracts PIASx-alpha from androgen receptor and eventually induces transcriptional activity of androgen receptor (Takahashi et al., 2001). As this experiment is conducted in a cell-free environment, the role of other potential effectors of HIF-1 ubiquitination is minimized.

Fourth, we demonstrated that accumulation of HIF-1α through genetic or pharmacological methods rescued DJ-1 deficiency- mediated hypersensitivity of neurons to MPP^+, suggesting DJ-1 acts in pathway with HIF-1 to protect neurons in an *in vitro* model of PD. To confirm the functional link between DJ-1 and HIF-1 in the context of cell survival, more investigation is needed on downstream targets of HIF-1 in DJ-1 over-expressing or deficient neurons. For instance, it would be interesting to examine whether over-expression or inhibition of downstream targets of HIF-1 such as VEGF or EPO, could affect the protective function of DJ-1. Our suggestion for the role of HIF-1 in PD is consistent with previous reports indicating the ability of HIF-1 and its downstream proteins to protect cells against MPTP toxicity in mice (Genc et al., 2001; Genc et al., 2002; McLeod et al., 2006; Puskovic et al., 2006; Lee et al., 2009a). Additionally down-regulation of the HIF-1 signaling pathway has been demonstrated in the SNc of human PD patients (Elstner et al., 2011). In line with the latter observation and our data, we showed that HIF-1 response to oxidative stress is also attenuated in DJ-1-linked human PD cases.

Our results strongly suggest that the DJ-1/VHL/HIF-1 pathway plays an important role in the pathogenesis of DJ-1 associated PD. Uncovering additional factors involved in the proposed pathway could give rise to a more comprehensive explanation of the function of DJ-1 in PD and even other diseases.

PI3K/ AKT pathway as a potential downstream effector of DJ-1/ VHL/ HIF-1 pathway

The PI3K/Akt is an established pathway, that when activated, protects cells against a number of cell death models, including oxidative stress (Chong et al., 2003; Kang et al., 2003; Chong et al., 2004; Aleyasin et al., 2010). We have previously shown that one of the downstream targets of DJ-1 in the context of PD is the PI3K/AKT pathway, where DJ-1 plays a central role in promoting phosphorylation of AKT in response to oxidative stress (Aleyasin et al., 2010) and potentially, activating the AKT downstream pathways. DJ-1, however, appears not to have any direct effect on PI3K or AKT, as it does not possess any detectable kinase activity, nor does it physically interact with PI3K or AKT. Thus, how DJ-1 induces the pro-survival AKT pathway remains elusive.

Interestingly, besides DJ-1, HIF-1 target genes EPO and VEGF have also been reported to up-regulate the AKT pathway and exert their protective roles against oxidative stress or hypoxia through this pathway (Kilic et al., 2005; Kilic et al., 2006; Wu et al., 2007c; Maiese et al., 2008; Burger et al., 2009; Zhong et al., 2012). For instance, it has been demonstrated that EPO induces phosphorylation of AKT by PI3K, which leads to inhibition of pro-apoptotic proteins glycogen synthase kinase-3β (GSK-3β) and caspase-3, downstream of AKT (Wu et al., 2007c). In addition, a protective role of EPO has been demonstrated in an MPP^+ model in PC12 cells, whereby it inhibits caspase-3 through PI3K/AKT/GSK3

pathway (Wu et al., 2007c).

By acting downstream of DJ-1/VHL/HIF-1 and upstream of PI3K/AKT pathways, EPO and VEGF are possible candidates for linking these two pathways together. Accordingly, lack of functional DJ-1, as observed in DJ-1 linked PD, could result in higher VHL activity, reduced HIF-1 stabilization, reduced EPO and VEGF induction, impaired PI3K/AKT response and eventually susceptibility of cells to oxidative damage.

This signalling scheme could also describe DJ-1 involvement in other disease conditions. For example, we have recently shown that DJ-1 is also a critical regulator of stroke-induced neuronal injury (Aleyasin et al., 2007). Lack of DJ-1 hypersensitizes neurons to excitotoxicity and ischemia, whereas DJ-1 over-expression can reverse this hypersensitivity and offer further protection (Aleyasin et al., 2007). Considering the DJ-1/VHL interaction, it is reasonable to infer that DJ-1 might protect neuronal cells from ischemic damage by inactivating VHL, leading to accumulation of HIF-1α, EPO and VEGF. Many lines of evidence have shown that these proteins play a central role in mediating ischemic stroke response and are potential therapeutic targets for recovery following stroke (Sun et al., 2003; Vezzani, 2008; Shi, 2009). More interestingly, at the biochemical level, both EPO and VEGF-mediated-response, like that of DJ-1 (Aleyasin et al., 2010), involve the PI3K/AKT pathways in this model (Kilic et al., 2005; Kilic et al., 2006). Our findings support a critical role of DJ-1/VHL/HIF-1 pathway in ischemic stroke response. Taken together, DJ-1 and its potential pathways, such as PON2, VHL/HIF-1 and PI3K/AKT, could be considered as therapeutic targets for PD or stroke.

Interestingly, the role of DJ-1 in survival may also have implications for diseases outside the CNS. For example, it is intriguing to speculate that excess DJ-1 mediated survival activity is deleterious in the context of tumorigenesis. Indeed, DJ-1 is an oncogene which transforms mouse NIH3T3 cells in co-operation with ras (Nagakubo et al., 1997) and has been found to be involved in breast cancer, lung cancer and prostate cancer (Nagakubo et al., 1997; Kim et al., 2005b; Tillman et al., 2007). Importantly, VHL protein has long been known as a tumour suppressor, whose loss of function leads to cancer (Maher et al., 1990; Latif et al., 1993; Lonser et al., 2003). This, at least to some extent, is via over-accumulation of HIF-1 and its downstream genes (Reifenberger et al., 1995; Clifford et al., 2001; Hoffman et al., 2001; Kondo et al., 2002). In addition, both VHL and DJ-1 have roles in renal cell carcinoma development (Sitaram et al., 2009). FInally, DJ-1 is a regulator of HIF-1 activity in cancer cells (Vasseur et al., 2009). Concurrently, VEGF acts as a stimulator of the AKT pathway in certain cancers (Trinh et al., 2009) and its signaling is reduced in absence of DJ-1 (Vasseur et al., 2009). Taken together, these data may suggest that the DJ-1/VHL/HIF-1 pathway is also involved in tumor formation via the pro-survival PI3K/AKT pathway.

In summary, we provide evidence that DJ-1 interacts with VHL and PON2 in mammalian cellular systems. These interactions inhibit VHL E3 ubiquitination function and induce PON2 pro-survival activity, as DJ-1 KO cells have increased VHL and reduced PON2 activity. Moreover, accumulation of the VHL target protein, HIF-1α, and expression of PON2 in DJ-1 deficient neurons is protective against in vitro model of PD.

In a broader view, we could consider a model in which DJ-1 plays an important role in balancing between pro-survival and pro-death events. In this model, DJ-1 provides survival through the VHL/HIF and PON2 pathways which, when regulated, favors survival of

neurons during times of oxidative stress or hypoxia. If unregulated, DJ-1 expression may lead to abnormal survival and subsequently, tumor genesis (Witte et al., 2011) by inhibiting the VHL-mediated tumor suppression function and inducing PON2 anti-apoptotic role. Interestingly, up-regulation of DJ-1 (He et al., 2012) and pro-survival role of PON2 (Witte et al., 2011) in cancer cells have been reported.

APPENDIX A: References for General introduction and General discussion

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APPENDIX B: Additional publications

I. DJ-1 protects the nigrostriatal axis from the neurotoxin MPTP by modulation of the AKT pathway.

Aleyasin H, Rousseaux MW, Marcogliese PC, Hewitt SJ, Irrcher I, Joselin AP, **Parsanejad M**, Kim RH, Rizzu P, Callaghan SM, Slack RS, Mak TW, Park DS.

Proceeding of the National Academy of Science USA. 2010 Feb 16;107(7):3186-91. Epub 2010 Jan 26.

II. Pim-1 kinase as activator of the cell cycle pathway in neuronal death induced by DNA damage.

Zhang Y, **Parsanejad M**, Huang E, Qu D, Aleyasin H, Rousseaux MW, Gonzalez YR, Cregan SP, Slack RS, Park DS.

J Neurochem. 2010 Jan;112(2):497-510. Epub 2009 Nov 6

III. Role of Cdk5-mediated phosphorylation of Prx2 in MPTP toxicity and Parkinson's disease.

Qu D, Rashidian J, Mount MP, Aleyasin H, **Parsanejad M**, Lira A, Haque E, Zhang Y, Callaghan S, Daigle M, Rousseaux MW, Slack RS, Albert PR, Vincent I, Woulfe JM, Park DS.

Neuron. 2007 Jul 5;55(1):37-52.

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Dear Sir/madam

My name is Mohammad Parsanejad, a PhD student at University of Ottawa, Canada. I am a co-author of a research article entitled : " DJ-1 protects the nigrostriatal axis from the neurotoxin MPTP by modulation of the AKT pathway ", published in PNAS in Feb 2010. As a part of my study, I need to include this paper in my PhD dissertation. Thus, I would like to ask for a permission of re-print for this article from PNAS.

Very much appreciate your consideration.

Best
Mohammad Parsanejad
PhD Candidate
Neuroscience research institute
University of Ottawa
451 Smyth Rd. Rm. 3522
Ottawa, ON
K1H 8M5

DJ-1 protects the nigrostriatal axis from the neurotoxin MPTP by modulation of the AKT pathway

Hossein Aleyasin[a,1], Maxime W. C. Rousseaux[a,1], Paul C. Marcogliese[a], Sarah J. Hewitt[a], Isabella Irrcher[a], Alvin P. Joselin[a], Mohammad Parsanejad[a], Raymond H. Kim[b], Patrizia Rizzu[c], Steve M. Callaghan[a], Ruth S. Slack[a], Tak W. Mak[b,2], and David S. Park[a,d,2]

[a]Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, ON K1H 8M5, Canada; [b]Campbell Family Institute for Breast Cancer Research, Toronto, ON M5G 2C1, Canada; [c]Section Medical Genomics, Department of Clinical Genetics, Vrije Universiteit University Medical Center, Van der Boechorststraat 7, 1081 BT, Amsterdam, The Netherlands; and [d]Department of Cogno-Mechatronics Engineering, Pusan National University, Geumjeong GU, Busan 609 735 South Korea

Contributed by Tak Wah Mak, December 24, 2009 (sent for review December 15, 2009)

Loss-of-function DJ-1 (PARK7) mutations have been linked with a familial form of early onset Parkinson disease. Numerous studies have supported the role of DJ-1 in neuronal survival and function. Our initial studies using DJ-1-deficient neurons indicated that DJ-1 specifically protects the neurons against the damage induced by oxidative injury in multiple neuronal types and degenerative experimental paradigms, both in vitro and in vivo. However, the manner by which oxidative stress-induced death is ameliorated by DJ-1 is not completely clear. We now present data that show the involvement of DJ-1 in modulation of AKT, a major neuronal pro-survival pathway induced upon oxidative stress. We provide evidence that DJ-1 promotes AKT phosphorylation in response to oxidative stress induced by H_2O_2 in vitro and in vivo following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment. Moreover, we show that DJ-1 is necessary for normal AKT-mediated protective effects, which can be bypassed by expression of a constitutively active form of AKT. Taken together, these data suggest that DJ-1 is crucial for full activation of AKT upon oxidative injury, which serves as one explanation for the protective effects of DJ-1.

neurodegeneration | Parkinson disease | reactive oxygen species

Individuals with homozygous loss-of-function mutations of DJ-1 (PARK7) have been clinically characterized with familial early onset Parkinson disease (PD) (1, 2). Although the physiological role of DJ-1 is not completely understood, several lines of evidence indicate a protective role for DJ-1 in multiple models of neuronal and nonneuronal oxidative stress-induced cell death (3–7). For example, we have previously shown that genetic ablation of DJ-1 in mice hypersensitizes dopamine neurons to the toxic effects induced by the mitochondrial toxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). This sensitivity was reversed by the induction of virally delivered human DJ-1 (8). These observations are in line with data by other groups showing sensitivity of dopaminergic neurons in DJ-1–deficient *Drosophila* models, as well as increased susceptibility to oxidative stress in vitro (9–11). To further support the importance of DJ-1 in managing oxidative stress, we provided evidence showing that DJ-1 protects the brain against ischemic injury that models clinical stroke. Moreover, our data indicated a direct correlation between DJ-1 neuroprotective activity and the reduced levels of oxidized DNA nucleotide species, 8-oxo guanine, a marker of oxidative damage (12).

Despite the fact that the neuroprotective role of DJ-1 has been consistently shown in multiple models of neurodegeneration, the exact mechanism of the neuroprotective function has not been fully elucidated. A direct antioxidant property of DJ-1 as a reactive oxygen species (ROS) scavenger has been proposed as a mechanism to overcome oxidative stress (7, 13). In fact, recombinant human DJ-1 confers some ROS scavenging activity; however, this activity is much weaker than any known peroxidase, thus not fully explaining its neuroprotective function (10, 13). Several alternative mechanisms to account for the neuroprotective function of DJ-1 have been suggested. For example, via its putative role in transcription regulation (14), DJ-1 up-regulates the expression of other antioxidant genes, such as glutathione synthase, during oxidative stress (15). Interestingly, it has also been reported that DJ-1 enhances the activity of the transcription factor Nrf2, a master regulator of antioxidant genes (16, 17). Alternatively, DJ-1 has also been shown to modulate key signaling pathways (3, 10). One signaling pathway implicated with DJ-1 function and relevant to the present work is AKT (10, 18).

AKT is a member of a larger class of serine/threonine kinases called AGC [protein kinase A (AMP protein kinase), PKG (GMP protein kinase), and PKC]. AKT has an N-terminus pleckstrin homology domain that mediates the interaction of AKT with a plasma membrane phospholipid, phophatidylinositol 3,4,5-triphosphate (PIP3). Extensive studies have shown that recruitment of AKT to the plasma membrane, and its association with PIP3, is crucial for its activation (19, 20). Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is particularly known for its action to convert PIP3 to phosphatidylinositol-4, 5- biphosphate (PIP2). This function of PTEN directly antagonizes PI3K to eventually down-regulate AKT (21, 22). Several lines of evidence indicated that the AKT signaling pathway responds to oxidative stress (23) and exerts a neuroprotective function (24, 25). Moreover, a large number of studies in vitro have illustrated that pharmacological compounds that protect cells against oxidative stress exert their neuroprotective effects through activation of the AKT pathway (26–30).

Early studies described DJ-1 as a negative regulator of PTEN using a *Drosophila* genetic screen (31). Evidence to confirm this negative regulation was demonstrated via down-regulation of DJ-1 using small interfering RNA, which resulted in the inhibition of endogenous AKT phosphorylation in cancer cell lines as well as in the *Drosophila* brain (10, 31, 32). Furthermore, loss of DJ-1 has been shown to reduce AKT activation in response to hypoxia in murine embryonic fibroblasts (MEFs) (33). However, the relevance of this pathway has yet to be shown in the context of neurons either in vitro or in vivo. Evidence to support a role for DJ-1 in the regulation of the AKT pathway would be particularly important when one considers the genetic linkage of DJ-1 to familial PD. Presently, we provide direct evidence, both in vitro and in vivo, that DJ-1 exerts an important role in the regulation of the AKT pathway in response to oxidative stress and neuronal protection. In

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The authors declare no conflict of interest.

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[1]H.A. and M.W.C.R. contributed equally to this work.

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addition, based on our results, we propose a mechanism suggesting that DJ-1 acts as an upstream regulator of AKT through membrane recruitment to confer neuroprotection.

Results

Phosphorylation of AKT in Response to Oxidative Stress Is Reduced in the Absence of DJ-1 in Vitro and in Vivo. To examine the role of DJ-1 on AKT signaling, we first determined whether lack of DJ-1 affects AKT phosphorylation following hydrogen peroxide (H_2O_2) treatment. To test this, neurons harvested from DJ-$1^{-/-}$ or DJ-$1^{+/+}$ embryos were treated with 100-μM H_2O_2 for indicated time points. As demonstrated in Fig. 1*A*, phosphorylation of AKT peaked in wild-type neurons at 15 min, whereas in the knockout there was a reduction in AKT phosphorylation. Quantification of three independent experiments revealed a significant reduction in p-AKT 15 min following treatment (3.67 ± 0.17 in DJ-$1^{+/+}$ vs. 1.49 ± 0.76 in DJ-$1^{-/-}$), as demonstrated in Fig. 1*B*. To further support this observation and to examine this response in a more clinically relevant model of PD, we examined AKT phosphorylation in dopaminergic neurons of the *substantia nigra* (SNc) in response to MPTP treatment. As indicated in Fig. 1*C*, and quantified in Fig. 1*D*, AKT phosphorylation in response to MPTP was reduced in the SNc cells of DJ-$1^{-/-}$ compared to wild-type controls (1.19 ± 0.10 vs. 1.52 ± 0.14, respectively). There was no significant increase in AKT phosphorylation when comparing saline and MPTP treated DJ-$1^{-/-}$ animals (1.00 ± 0.2 vs. 1.19 ± 0.10, respectively). To further confirm these results, we also examined AKT phosphorylation in response to H_2O_2 in human lymphoblasts from human PD patients harboring DJ-1 mutations. As demonstrated in Fig. 1*E*, AKT response was significantly attenuated in L166P mutated cells compared to the controls.

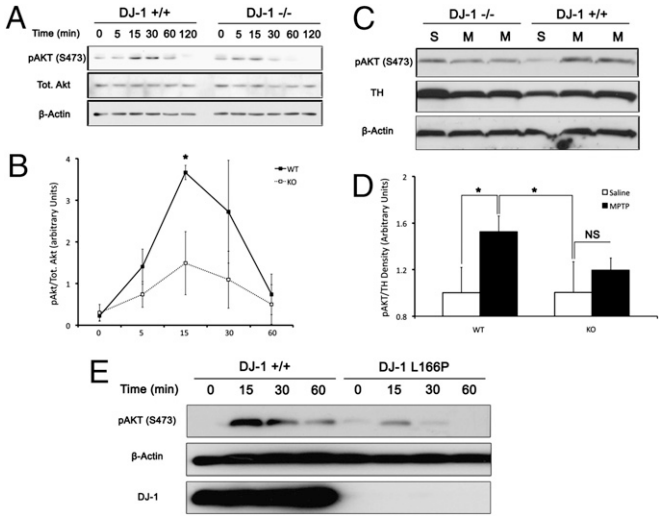

Fig. 1. AKT activation is suppressed in the absence of DJ-1. (*A*) Cortical neurons from DJ-$1^{+/+}$ and DJ-$1^{-/-}$ embryos were harvested, plated, and treated with H_2O_2 (100 μM) in a time-dependent fashion. Extracts were probed for pAKT (S473), total AKT, and β-actin by Western blot. (*B*) Quantification of *A* from three independent experiments. Values are presented as mean optical density relative to total AKT. (*C*) Eight- to 10-week-old C57Bl6 mice of WT and DJ-1 knockout genotype were treated with two 30-mg/kg doses of MPTP (M), or saline (S), given 24 h apart. Three hours following the second injection, mice brains were quickly dissected for SNc and samples were processed for Western blot analysis. (*D*) Quantification of *C*. $n = 3$–6 per group. (*E*) Immortalized lymphoblasts derived from patients with the DJ-1 L166P mutation or healthy control lymphoblasts were treated with H_2O_2 in a time dependant manner. Analysis of cell lysates was carried out by Western blot. Blot presented is representative of two independent experiments. Data are presented as mean ± SEM.

DJ-1 Is Necessary for AKT-Mediated Neuroprotective Function in Vitro and in Vivo. We next evaluated the functional role of DJ-1 in the protective effects of AKT following oxidative stress. First, we examined the role of AKT in protecting neurons against oxidative stress induced by H_2O_2 in vitro. Neurons, transfected with HA-tagged wild-type AKT along with GFP expression vectors as a marker of transfection (or GFP/empty vector transfection as control) were treated with H_2O_2, 24 h after transfection, and survival was assessed as described in *Materials and Methods* (Fig.2*A*). As shown in Fig. 2*B*, induction of exogenous wild-type AKT confers protection in DJ-$1^{+/+}$ neuronal cells in response to H_2O_2. Next, DJ-$1^{-/-}$ cortical neurons were tested to examine whether induction of wild-type AKT could provide similar protection in DJ-1-deficient cells. Surprisingly, induction of exogenous AKT failed to protect DJ-$1^{-/-}$ neurons against H_2O_2-induced death (Fig. 2*C*). To confirm these observations, we cultured neurons harvested from DJ-$1^{-/-}$ and DJ-$1^{+/+}$ litters at the same time. Three days after plating, the cells were transiently transfected with wild-type AKT together with or without a DJ-1

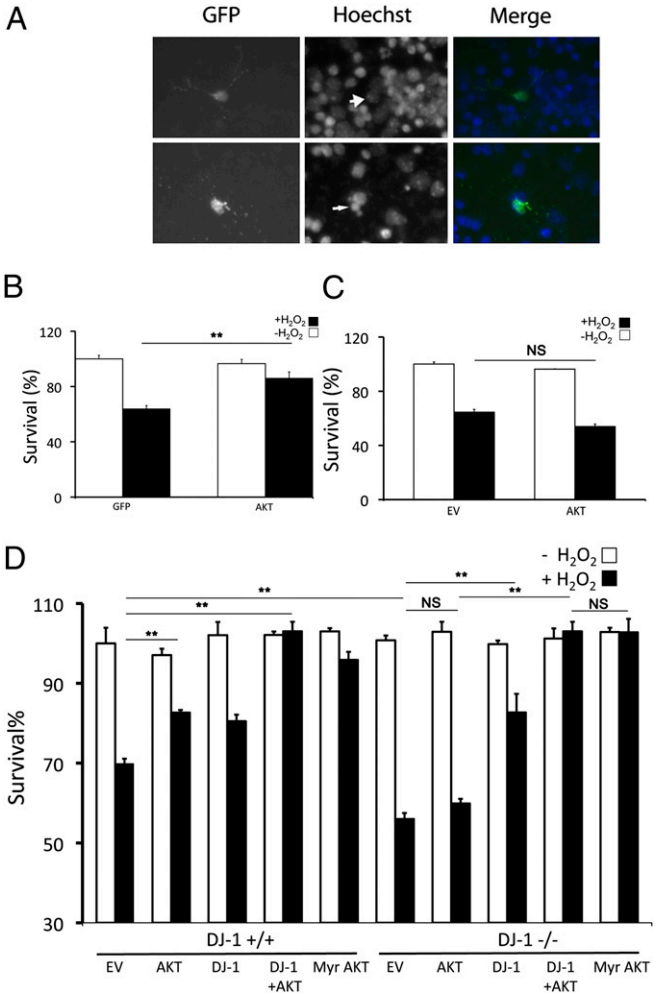

Fig. 2. AKT requires DJ-1 to exert its neuroprotective function in vitro. (*A*) Representative pictures of alive (*Upper*, large arrowhead) and dead (*Lower*, thin arrowhead) neurons. Neuronal survival was measured by identifying GFP-positive cells and determining their nuclear integrity by Hoechst stain. (*B–D*) Cortical neurons from either DJ-$1^{+/+}$ or DJ-$1^{-/-}$ embryos were harvested, plated, and transfected with empty vector (EV), AKT, DJ-1, or Myr AKT. Cells were treated with H_2O_2 (30 μM) or vehicle control ($-H_2O_2$) for 3 h. Quantification was assessed as in *A*. Data are presented as mean ± SEM. **, $P < 0.01$; NS, no significant difference.

expression vector, DJ-1 only, or myristoylated AKT, a membrane-anchored constitutively active form of AKT (34). After treatment with H_2O_2, cell survival was assessed. The results of this experiment clearly verified our findings in Fig. 2*C*, indicating the protective role of wildtype AKT expressed in DJ-$1^{+/+}$ neurons but not in DJ-$1^{-/-}$ cells (82.65 ± 0.65% DJ-$1^{+/+}$ vs. 59.88 ± 1.18% DJ-$1^{-/-}$) (Fig. 2*D*). Interestingly, myristoylated AKT significantly protects neurons against oxidative damage induced by H_2O_2 regardless of DJ-1 genotype (95.85 ± 2.02% DJ-$1^{+/+}$ vs. 102.77 ± 3.38% DJ-$1^{-/-}$).

Suppression of AKT Abolishes the Neuroprotective Function of DJ-1 in Vitro and in Vivo. The observations that DJ-1 deficiency reduces AKT activation and that wild-type AKT requires DJ-1 to effectively protect neurons against oxidative stress suggests DJ-1 acts as an upstream activator of AKT. We next determined whether DJ-1 exerts its neuroprotective effects, at least partially, through the AKT pathway. To examine this, we first used a conventional pharmacological inhibitor of AKT, LY294002 (LY) (35). Because the basal activity of AKT is essential for the long-term health of cultured neurons, we determined the optimal dose of inhibitor that suppressed AKT with minimal toxicity to the cultured neurons (Fig. 3*A*). We next infected cultured cortical neurons with adenoviral vectors expressing GFP only or DJ-1 and GFP on separate promoters at the time of plating. Thirty-six hours after plating, we pretreated the cells with 10-μM LY or vehicle for 30 min before application of H_2O_2 or vehicle for 3 h. Cells were then assessed for survival. As shown in Fig. 3*B*, the neuroprotective activity of DJ-1 is significantly reduced upon suppression of AKT phosphorylation by LY (52.78 ± 0.20% vs. 40.55 ± 0.55%, respectively). We also used a more specific molecular strategy to validate our results by transiently transfecting a phosphorylation mutant, dominant-negative form (DN-AKT) of AKT (AAA-AKT) into cortical neurons. In this mutant, all phosphorylation sites of AKT have been mutated to alanine; therefore, this artificial mutant of AKT is incapable of being phosphorylated and displays dominant-negative properties toward endogenous AKT (21). As shown in Fig. 3*C*, the results of this experiment confirmed that suppression of AKT diminished the neuroprotective function of DJ-1 (84.46 ± 2.90% without DN-AKT vs. 59.26 ± 2.01% with DN-AKT).

DJ-1 Is Necessary for AKT-Mediated Neuroprotection in Vivo Following MPTP Treatment. In vitro experiments indicated that DJ-1 is necessary for AKT activation and is neuroprotective in response to H_2O_2. To confirm these results and to test this hypothesis in a more clinically relevant paradigm, we examined whether induction of wild-type AKT can protect nigrostriatal neurons against the dopaminergic specific neurotoxin MPTP in vivo. To achieve this, we injected adenoviral vectors harboring HA-tagged wild-type AKT or myristoylated AKT into the striatum of DJ-$1^{+/+}$ and DJ-$1^{-/-}$ age-matched mice. β-gal expressing adenoviruses

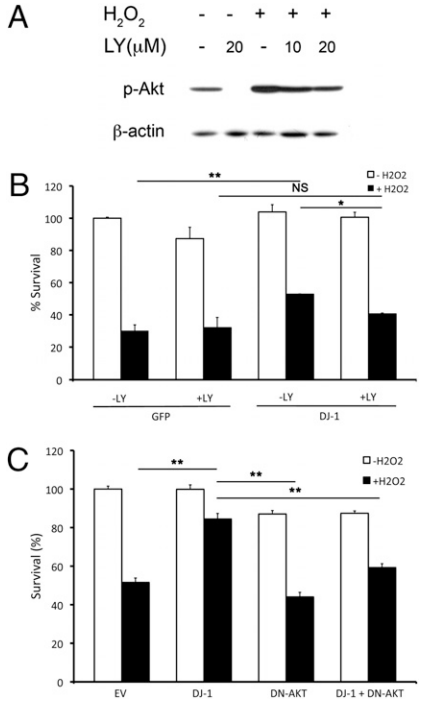

Fig. 3. DJ-1 requires AKT activation to promote cellular survival in vitro. (*A*) Cortical neurons were treated for either 10 or 20 μM of LY with and without H_2O_2 (100 μM, 15 min) to determine the effective dose of LY. (*B*) Cortical neurons were infected with either GFP or DJ-1 with GFP. Cells were then pretreated with LY followed by H_2O_2 treatment for survival assessment. (*C*) Cortical neurons were cotransfected with GFP and empty vector (EV), DJ-1, DN-AKT, or a DJ-1/DN-AKT combination followed by H2O2 treatment. Survival was assessed as in *B*. Data are presented as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; NS, no significant difference.

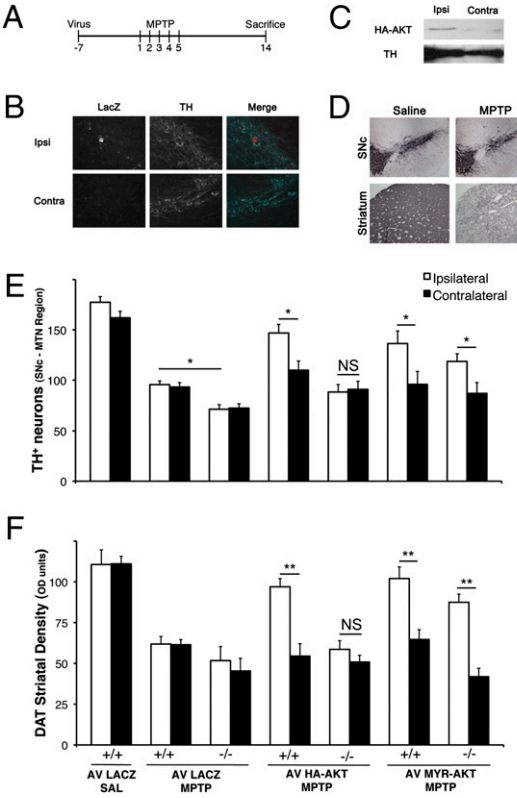

Fig. 4. AKT requires DJ-1 to exert its neuroprotective function in an in vivo model of PD. (*A*) Schematic representation of treatment course. Mice were injected ipsilaterally in the striatum with adenovirus (LacZ, HA-AKT, Myr-AKT) 7 d before commencement of MPTP injections. MPTP was injected for 5 consecutive days and brains were collected 14 days following the first MPTP injection. (*B*) Confirmation of virus expression was performed by immunohistochemistry. Dual labeling of both TH and protein of interest in the SNc. (*C*) HA expression was tested in the SNc by Western blot analysis. (*D*) Representative pictures of both striatum and SNc of mice treated with MPTP or saline. SNc and striatum were stained for TH and DAT, respectively. (*E*) Quantification of TH-immunoreactive neurons was performed at the MTN region of the SNc where virus expression was highest. "Ipsi" denotes the side of the brain ipsilateral to the virus injection, whereas "contra" denotes the contralateral side. (*F*) Quantification of DAT-positive fibers normalized to cortex (DAT-negative). Data are presented as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; NS, no significant difference.

were used as control. As shown in Fig. 4 *B* and *C*, the virus localizes specifically to the ipsilateral side in dopamine neurons. One week after virus injection, we performed a subchronic MPTP treatment paradigm as indicated in Fig. 4*A*. Two weeks after the initial MPTP injection, animals in all groups were sacrificed and prepared for histological analysis. We first assessed survival by counting the number TH+ neurons of SNc at the level of the medial terminal nucleus (MTN) (Fig. 4*E*). Consistent with our in vitro observations, DJ-$1^{+/+}$ animals that received wild-type HA-tagged AKT and were subjected to MPTP treatments showed larger number of surviving TH+ neurons in the ipsilateral side of virus injection, compared to the contralateral side (146.9 ± 8.5 vs. 109.9 ± 9.2, respectively). Meanwhile, there was no significant difference between ipsi- and contralateral sides of the SNc in the knockout animals that received HA-AKT. (88.2 ± 7.4 vs. 90.9 ± 8.0, respectively). To verify whether the loss of TH immunoreactivity was in fact a result of the death of dopaminergic neurons and not loss of expression, we stained for cresyl violet and assessed neuronal survival in the MTN region of the SNc. Similarly, a substantial rescue was observed in the wild-type mice (62.8 ± 1.9% vs. 50.7 ± 3.0%, ipsilateral vs. contralateral), whereas no protective effect was observed when injecting HA-AKT in the DJ-$1^{-/-}$ mice (48.9 ± 3.0% vs. 51.9 ± 4.0% ipsilateral vs. contralateral) (Fig. S1). To further substantiate the SNc neuronal survival results, we examined whether prophylactic administration of virus could rescue dopaminergic terminals in the striatum of the animals subjected to MPTP injection in each group using expression of dopamine transporter (DAT) as a marker of dopaminergic terminals. Consistent with SNc results, higher densities, and thus greater survival of dopaminergic terminals, were observed following MPTP treatment in the striatum of virus-injected sides compared to the contralateral sides in the AKT-expressing group (97.0 ± 5.0 vs. 54.5 ± 7.6 for HA-AKT, respectively) (Fig. 4*F*). Such protection was not observed in DJ-$1^{-/-}$ animals, which signifies that the AKT-mediated neuroprotection is dependent upon the presence of DJ-1. In line with our observations in vitro and in vivo, myristoylated AKT (Myr-AKT) provides protection to both DJ-$1^{-/-}$ and DJ-$1^{+/+}$ animals (87.5 ± 5.0 ipsilateral vs. 41.9 ± 5.2 contralateral, and 102.0 ± 7.1 ipsilateral vs. 64.7 ± 6.0 contralateral, respectively). All viruses were also injected without MPTP treatment to note effects of virus toxicity. No significant death of SNc neurons was attributed to viral vectors.

DJ-1 Modulates AKT Translocation to Membranous Fractions. Our results in vitro and in vivo demonstrated specifically that myristoylated rather than a wild-type form of AKT promotes protection in DJ-$1^{-/-}$ neurons. We therefore tested whether DJ-1 was affecting AKT translocation to membranous compartments following oxidative stress. This was done by determining the subcellular localization of AKT following H_2O_2 treatment in DJ-$1^{+/+}$ and DJ-$1^{-/-}$ neurons and MEFs. As shown in Fig. 5*A*, the H_2O_2-induced AKT localization to the membrane fraction is greatly decreased in the DJ-$1^{-/-}$ compared with the DJ-$1^{+/+}$ MEFs. Quantification revealed AKT translocation to the membrane fraction following treatment that was 4-fold greater in the DJ-$1^{+/+}$ than in DJ-$1^{-/-}$ cells (2.94 ± 1.14 vs. 0.78 ± 0.30, respectively). Similarly, in DJ-$1^{-/-}$ neurons, no AKT translocation was observed following H_2O_2 (0.58 ± 0.10-fold increase), whereas DJ-$1^{+/+}$ neurons showed an AKT translocation 5 min post-treatment (1.55 ± 0.37 fold increase) No significant differences were observed in levels of total AKT in the cytoplasmic fraction.

Discussion

DJ-1 was first discovered as a weak oncogene with an unclear mechanism of action (36). Since then, putative roles for DJ-1 have been proposed, which include functions in transcriptional regulation either via binding to and modulating an androgen receptor inhibitor, PIASx-alpha (37), as well as RNA–protein

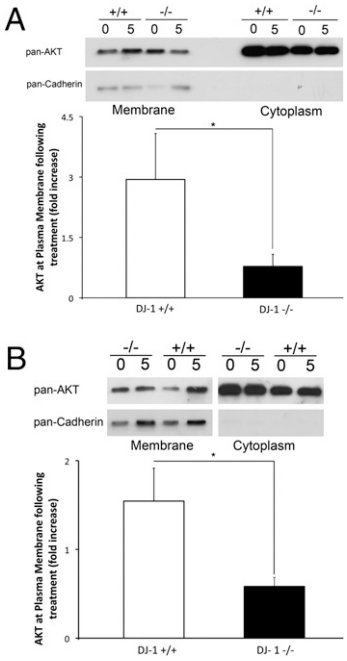

Fig. 5. AKT requires DJ-1 to localize to membranous fractions following oxidative insult. (*A*) DJ-$1^{+/+}$ and DJ-$1^{-/-}$ MEFs were treated with 500 μM of H_2O_2 for 5 min or with media control. Western blot analysis by probing pan-AKT and pan-Cadherin as membranous fraction control. Quantification of membranous fractions was performed in the lower panel by calculating relative AKT density normalized to Cadherin levels and normalizing treatment group to control. Data are representative of $n = 4$ experiments. (*B*) DJ-$1^{+/+}$ and DJ-$1^{-/-}$ cortical neurons were subjected to 100 μM of H_2O_2 for 5 min or media control. Quantification was performed as in *A*. Data are presented as mean ± SEM. *, $P < 0.05$.

interactions (38). The DJ-1 protein also displayed some homology to the proteins of the ThiJ/PfpI family of bacterial proteases (39, 40), suggesting a putative chaperone function. Interestingly, DJ-1 was also noted to display an isoelectric pH shift upon induction of oxidative stress, potentially placing DJ-1 within the oxidative stress-response pathway. Despite these important implications, its physiological relevance was not entirely clear until its genetic linkage to PD. To this end, several themes regarding DJ-1 have now emerged that link this protein to neurodegeneration, PD, and oxidative stress. These themes include, but are not limited to the following: (*i*) DJ-1 protects neurons against oxidative stress (4, 9, 11, 41–44); (*ii*) Loss of DJ-1 on its own does not lead to dopamine neuron death, at least in mice, but DJ-1-deficient animals are sensitized to environmental stress and exhibit impaired dopamine signaling (45–48); and (*iii*) DJ-1 modulates signaling pathways critical to cell survival such as PTEN and AKT, at least in select nonneuronal contexts (31).

In the present study, we more carefully characterized the necessity of DJ-1 for activation of the AKT pathway in response to oxidative injury, particularly in neurons. We first demonstrated that the absence of DJ-1 significantly attenuates AKT phosphorylation in vitro and in vivo, as well as in human lymphoblasts derived from PD patients harboring pathogenic DJ-1 mutations. Importantly, even though AKT phosphorylation is not completely abolished by loss of DJ-1, we also demonstrated that the significant attenuation in AKT signaling brought about by DJ-1 deficiency resulted in enhanced cell death both in vitro and in vivo. These data not only highlighted an important functional role for DJ-1 in AKT-mediated cell survival, but also indicated that the AKT pathway is integral to the mechanism of protection conferred by DJ-1 and suggested that DJ-1 could be an upstream regulator of AKT.

In light of our findings above, together with the knowledge that AKT is considered to be part of the survival pathway, we sought to further investigate the nature of the DJ-1/AKT relationship. We first demonstrated that overexpression of AKT alone protects cultured neurons exposed to oxidative stress in vitro as well as dopamine neurons exposed to MPTP in vivo. Furthermore, inhibition of the PI3K/Akt pathway significantly reduces the protection that is conferred by DJ-1. Importantly, we also demonstrated that wild-type AKT required DJ-1 to exert its protective effect as DJ-1 deficiency abrogated the effect of AKT on cell survival. Interestingly, the protective effects of AKT in a DJ-1-deficient background can be bypassed using myristoylated AKT, its membrane-anchored constitutively active form. This latter observation is consistent with reports that membrane-bound AKT is sufficient to provide protection following MPP^+ treatment both in vitro (49) as well as 6-OHDA treatment in vivo (50). Because AKT recruitment to the membrane is a prior event to its phosphorylation and activation (51, 52), these results, in addition to the cell fractionation experiments presented in our study, suggested that DJ-1 permits AKT translocation to the membrane fractions.

Our study therefore proposes a working model in which DJ-1 acts upstream of AKT, thereby facilitating its activation following neuronal injury via oxidative stress. We propose that DJ-1 may be involved in fine-tuning the response of neurons to ROS and modulation of signaling pathways that mediate survival. In this regard, it will be critical in future studies to address the possible mechanisms underlying the ROS-mediated, DJ-1-dependent activation of AKT. One might consider the possibility that DJ-1 regulates AKT by modulating its recruitment to the membrane in a ROS-dependent manner. It is noteworthy that the AKT response to H_2O_2 can be altered depending on antioxidant protein activity within the cell (53–55). Thus, further studies in models that permit well-controlled ROS levels are needed to address these questions. However, other possibilities exist. For example, a recent study has suggested that DJ-1 interacts with PTEN to permit AKT activation, although this needs to be further investigated in more physiologically relevant models (56). Additionally, DJ-1 may interact with other PI3K pathway kinases, such as mTOR and PDK, to permit AKT phosphorylation. Finally, while DJ-1 plays a significant role in facilitating AKT phosphorylation, other factors may also each play a role (21, 57, 58). Thus, additional studies should be performed to investigate the nature of the DJ-1/AKT interdependence.

Finally, it is interesting to note that even though it is clear that DJ-1 is linked to familial PD, there is a report of an epidemiological association with certain haplotype of AKT1 and a reduced risk of PD (59). This observation provides further strength to the notion that the DJ-1/AKT signaling axis may be important in regulating dopaminergic function or death. Elucidation of these mechanisms may provide an eventual basis for neuroprotective therapies.

Materials and Methods

Cell culture, Western blot analysis, and in vivo stereotaxic injections and MPTP administration were performed as previously described (60). All procedures involving animals were approved by the University of Ottawa Animal Care Committee and were maintained in strict accordance with the Guidelines for the Use and Treatment of Animals put forth by the Animal Care Council of Canada and endorsed by the Canadian Institutes of Health Research. For additional in vivo and in vitro procedures, see *SI Materials and Methods.*

Subcellular Fractionation. Membrane fractions were obtained similarly for MEFs and DIV 6 cortical neurons using differential centrifugation. Briefly, cells were harvested in cold PBS and centrifuged at 1,200 × *g* for 3 min. The cell pellet was resuspended in 200 μL of hypo-osmolar buffer [50 mM Tris-HCl, pH 7.4; 50 mM NaCl; protease inhibitor complex (Roche)] and homogenized for 30 s. Samples were centrifuged at 20,000 × *g*, at 4 °C for 20 min. Supernatants (cellular debris) were transferred to 1.5-mL ultracentrifuge tubes (Beckman) and centrifuged at 100,000 × *g*, at 4 °C for 3 h. The pellets (microsomal enriched) were resuspended in RIPA buffer (150 mM NaCl; 1% Nonidet P-40; 0.5% deoxycholic acid; 0.1% SDS; 50 mM Tris-HCl, pH 8.0) and sonicated briefly for subsequent Western blot analysis. Supernatants from final spin were used as a cytoplasmic control.

Statistical Analysis. Statistical significance was either determined by Student's *t*-test or one-way ANOVA followed by Tukey's post hoc test. All data are presented as mean ± SEM. Significance at $P < 0.05$ (*) and $P < 0.01$ (**), and NS denotes no significant difference.

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Pim-1 kinase as activator of the cell cycle pathway in neuronal death induced by DNA damage

Yi Zhang,* Mohammad Parsanejad,* En Huang,* Dianbo Qu,* Hossein Aleyasin,* Maxime W. C. Rousseaux,* Yasmilde Rodriguez Gonzalez,* Sean P. Cregan,† Ruth S. Slack* and David S. Park*

*Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, ON, Canada

†Robarts Research Institute and Department of Physiology and Pharmacology, University of Western Ontario, London, ON, Canada

Abstract

DNA damage is a critical component of neuronal death underlying neurodegenerative diseases and injury. Neuronal death evoked by DNA damage is characterized by inappropriate activation of multiple cell cycle components. However, the mechanism regulating this activation is not fully understood. We demonstrated previously that the cell division cycle (Cdc) 25A phosphatase mediates the activation of cyclin-dependent kinases and neuronal death evoked by the DNA damaging agent camptothecin. We also showed that Cdc25A activation is blocked by constitutive checkpoint kinase 1 activity under basal conditions in neurons. Presently, we report that an additional factor is central to regulation of Cdc25A phosphatase in neuronal death. In a gene array screen, we first identified Pim-1 as a potential factor up-regulated following DNA damage. We confirmed the up-regulation of Pim-1 transcript, protein and kinase activity following DNA damage. This induction of Pim-1 is regulated by the nuclear factor kappa beta (NF-κB) pathway as Pim-1 expression and activity are significantly blocked by siRNA-mediated knockdown of NF-κB or NF-κB pharmacological inhibitors. Importantly, Pim-1 activity is critical for neuronal death in this paradigm and its deficiency blocks camptothecin-mediated neuronal death. It does so by activating Cdc25A with consequent activation of cyclin D1-associated kinases. Taken together, our results demonstrate that Pim-1 kinase plays a central role in DNA damage-evoked neuronal death by regulating aberrant neuronal cell cycle activation.

Keywords: Cdc25A, cell cycle, DNA damage, neuron, NF-κB, Pim-1.

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Substantial evidence suggests that neuronal apoptosis evoked by DNA damage may be a critical event in neurodegenerative disease and stroke. DNA lesions have been widely reported in the pathogenesis of Parkinson's disease (Robison and Bradley 1984; Alam *et al.* 1997; Jenner 1998), Alzheimer's disease (Robison and Bradley 1984; Gabbita *et al.* 1998; Lovell and Markesbery 2001) and stroke (Tobita *et al.* 1995; Chen *et al.* 1997; Cui *et al.* 2000). Furthermore, defective response to DNA damage can lead to severe neurodegeneration (Deans *et al.* 2000; Frank *et al.* 2000; Culmsee *et al.* 2001; Vemuri *et al.* 2001). However, the mechanisms by which DNA damage triggers neuronal death in these neurological disorders are not fully understood.

Previous studies have shown that DNA damage in neurons induces death signals that involve aberrant activation of multiple cell cycle elements (Becker and Bonni 2004). For example, in cultured embryonic neurons treated with DNA damaging agent camptothecin, cyclin D1-associated kinases (Cdk4/6) are rapidly activated (Park *et al.* 1996, 1998), which results in phosphorylation of Rb family members and subsequent release and activation of E2F transcription factors (Park *et al.* 2000). Over-expression of dominant-negative Cdk4/6, over-expression of Rb or loss of E2F activity

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Address correspondence and reprint requests to David S. Park, Department of Cellular and Molecular Medicine, University of Ottawa, 451 Smyth Road, Ottawa, ON, Canada K1H 8M5.

Abbreviations used: ATM, ataxia telangiectasia mutated; CAPE, caffeic acid phenethlester; Cdc, cell division cycle; Cdk, cyclin-dependent kinase; Chk1, checkpoint kinase 1; IP, immunoprecipitation; JNK, c-*Jun* N-terminal kinase; NF-κB, nuclear factor kappa beta; PP2A, protein phosphatase 2A; SDS, sodium dodecyl sulfate.

protects cortical neurons from death attributable to DNA damage (Park *et al.* 1998, 2000). These observations are quite intriguing and unexpected given that neurons are terminally differentiated and should not utilize any cell cycle signals.

The mechanism by which abnormal cell cycle signals are activated in neurons is not fully understood. Recent reports have implicated checkpoint kinase 1 (Chk1) and cell division cycle (Cdc) 25A phosphatase as mediators of Cdk/Rb/E2F pathway in this death paradigm (Zhang *et al.* 2006). Camptothecin exposure leads to rapid inactivation of Chk1 and subsequent activation of Cdc25A which in turn activates Cdk4/6. Moreover, over-expression of Cdc25A promotes neuronal death evoked by camptothecin while siRNA-mediated knockdown of Cdc25A or over-expression of Chk1 blocks death (Zhang *et al.* 2006).

Despite progress on elucidating this cell cycle signaling pathway, the mechanisms underlying Cdc25A activation is not completely understood. For example, in proliferating cells exposed to genotoxic stress, Chk1-mediated Cdc25A inactivation occurs through phosphorylation-dependent degradation of Cdc25A (Sanchez *et al.* 1997; Mailand *et al.* 2000; Sorensen *et al.* 2003). However, in post-mitotic neurons, Cdc25A protein level does not change significantly upon DNA damage. This suggests that there may be other regulators of Cdc25A activation in addition to Chk1 critical for its activation. To address this issue, we recently performed a preliminary gene array screen to examine for increased message in cortical neurons exposed to camptothecin. One potential candidate of interest that we identified was Pim-1.

Pim-1 is a proto-oncogene which encodes a serine/threonine protein kinase that regulates cell proliferation and growth. It was originally identified as a preferential proviral insertion site in Moloney Murine Leukemia Virus induced T-cell lymphomas (Cuypers *et al.* 1984). Pim-1 plays important roles in signal transduction in hematopoietic and lymphoid systems (Wang *et al.* 2001; Bachmann and Moroy 2005). In addition, dysregulated expression or function of Pim-1 is associated with hematopoietic neoplasia (Amson *et al.* 1989; Saris *et al.* 1991; Laird *et al.* 1993; Lilly *et al.* 1999). Critically, it promotes cell survival in dividing cells consistent with its oncogenic properties (Lilly *et al.* 1999). These findings led us to examine whether Pim-1 signals camptothecin-mediated neuronal death and cell cycle activation. Presently, we show that Pim-1 activity in neurons promotes death, unlike that in dividing cells. In addition, we show that Pim-1 is critical for Cdc25A activation in neurons following DNA damage. This finding provides crucial understanding to how abnormal cell cycle signals are inappropriately activated in neurons. Finally, we show that nuclear factor kappa beta (NF-κB) is essential for the activation of Pim-1 in neurons exposed to DNA damage thereby providing a mechanism of how Pim-1 itself is activated.

Experimental procedures

Materials

Camptothecin and staurosporine were purchased from Sigma (St. Louis, MO, USA). Helenalin, BAY 11-7082 and CAPE (caffeic acid phenethlester) were obtained from Biomol (Plymouth Meeting, PA, USA). 4-Nitrophenol phosphate (pNPP) was purchased from Roche Applied Science (Indianapolis, IN, USA).

Neuronal cultures and transfection

Primary cortical neurons were cultured from embryonic day 15 (E15) CD1 mice (Charles River Laboratories, Wilmington, MA, USA) as previously described (O'Hare *et al.* 2005; Haque *et al.* 2008) and maintained in Neurobasal media (Invitrogen, Carlsbad, CA, USA) supplemented with B27 (Invitrogen), N2 (Invitrogen), and 0.5 mM glutamine (Sigma). 2 days after plating cells were treated with camptothecin (10 μM) or NF-κB inhibitors as indicated in the text and figures. Lipofactamine mediated transfection of siRNA for NF-κB have been described previously (Aleyasin *et al.* 2004). siRNAs used in Fig. 7 to (siRNAp65, AAGAAGCACAGA-UACCACCAA) and (control siRNA, GCGCGCUUUGUAGGA-UUCG) were obtained from Ambion (Austin, TX, USA) and siRNAs used in the Fig. S2 was a mixture of three targeted siRNA from Santa Cruz Biotechnology (Santa Cruz, CA, USA) (Cat.#, sc-29411). Pim-1 plasmid was transfected using calcium phosphate method as previously mentioned (Zhang *et al.* 2006).

RT–PCR

Total RNA was extracted from cultured cortical neurons using TRIzol reagent (Invitrogen). 50 ng of total RNA was used for cDNA synthesis and targeted gene amplification using SuperScript One-Step RT-PCR kit (Invitrogen). After DNase treatment, cDNA synthesis was performed at 45°C for 45 min followed by a 2-min initial denaturation step at 94°C. This was followed by 29 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min. The mouse-specific Pim-1 PCR primers (5′-GGCGCCGGGCAAAGAGAAG-GAG-3′ and 5′-ACCCGAAGTCGATGAGTTTGATTT-3′) were used to generate a 491-bp product. Expression of ribosomal S12 mRNA was used as a standard to quantify the relative amount of expression of Pim-1 as described previously (Cregan *et al.* 2004). S12 cDNA was amplified using 5′-GGAAGGCATAGCTGCTGG-3′ and 5′-CCTCGATGACATCCTTGG-3′ as primers.

Western blot analysis

Western blot analysis was performed as previously described (O'Hare *et al.* 2005) using antibodies against Pim-1 (Cat.# sc-13513; 1 : 1000; Santa Cruz Biotechnology) and β-actin (Cat.# A5316; Sigma) as a loading control. For Pim-1/Cdc25A binding assay, a rabbit anti-Cdc25A (Cat.# 07-459; Upstate, Lake Placid, NY, USA) and mouse anti-Pim-1 antibody mentioned above were used for immunoprecipitation of Cdc25A and Pim-1, respectively. A normal rabbit IgG (Cat.# sc-2027; Santa Cruz Biotechnology) and a mouse anti-GST IgG (Cat.# sc-138; Santa Cruz Biotechnology) were used as controls. The anti-Pim-1 antibody mentioned above and a rabbit anti-Cdc25A antibody (Cat.# sc-97; Santa Cruz Biotechnology) were used to detect immunoprecipitated Pim-1 and Cdc25A, respectively. Anti-p65 antibody (Cat.# sc-372; Santa Cruz Biotechnology) was used for p65 knockdown experiments.

Protein kinase assay
As previously reported (Appendix S1).

Immunofluorescence
As previously reported (Appendix S1).

Knockout mice
Pim-1 knockout mice (Laird *et al.* 1993) were maintained on a mixed C57BL/6 × 129/Ola background and genotyped as previously reported (Appendix S1).

Cdc25A phosphatase assay
As previously reported (Appendix S1).

Chromatin immunoprecipitation
Embryonic day 15 mouse cortical neurons at 3 DIV were incubated with 1% formaldehyde with mild rocking for 10 min at 22°C. Cells were washed with phosphate-buffered saline and harvested and resuspended in 200 μL lysis buffer [1% sodium dodecyl sulfate (SDS), 10 mm EDTA, 50 mm Tris/HCl, pH 8.1] containing protease inhibitors and placed on ice for 10 min. Cell lysates were sheared by sonication for a total of 50–100 s at 10 s per interval and centrifuged at 15 000 *g* at 4°C for 10 min and the supernatant was diluted at 1 : 10 in dilution buffer (0.01% SDS, 1% Triton X-100, 2 mm EDTA, 20 mm Tris/HCl, pH 8.1, 150 mm NaCl). 20 μL of the sample were used as the input chromatin. Immunoprecipitation was performed overnight with p65 antibody and rabbit IgG mentioned above, then 50 μL protein A agarose beads was added for 1 h at 4°C. Beads were washed three times with wash buffer (100 mM Tris-HCl, pH 9.0, 500 mM LiCl, 0.1% NP-40 (Sigma, St. Louis, MO, USA), and 1% deoxycholic acid). Bead precipitates were eluted twice with elution buffer (1% SDS 0.1 m NaHCO3). Formaldehyde cross-linking was reversed by heating overnight at 65°C, and incubating 1 h with RnaseA at 37°C, and incubating 1 h with proteinaseK at 37°C. DNA were isolated with phenol/chloroform and subjected to PCR reaction. The primers used for PCR amplification of mouse Pim-1 promoter region are 5′-AACCTAGGATTTTCTTCTGT-3′ and 5′-CATGCACCCTGTCACCA-3′. Human promoter sequences are also as follows: 5′-AGCCGACTTGGAGGTCTCGGGT-3′ and 5′-GCTGTAGGCTGGGGGCGGATTCTG-3′. PCR products were resolved on an agarose gel and photographed.

Luciferase assay
HEK293 cells were transfected using Lipofactamine 2000 with a luciferase vector (pGL4.23; Promega, Madison, WI, USA) containing a minimal promoter with or without the Pim-1 promoter sequence (−1261 to −23) along with plasmid cytomegalovirus vector (pCMV) (empty or expressing p65) and plasmid cytomegalovirus vector (pCMV)-LacZ 24 h later, cells were washed with cold phosphate-buffered saline, triturated off the plates, and pelleted in microcentrifuge tubes. Cell pellets were lysed in buffer provided in the Promega Luciferase System (Promega). Luciferase assay was carried out according to the manufacturer's instructions. Relative luciferase activities were obtained by normalizing the luciferase activity against β-gal activity.

Recombinant adenovirus infection
As previously reported (Appendix S1).

Results

To examine the signaling pathways induced by DNA damage, we have used the *in vitro* model of cortical neuronal death evoked by the DNA topoisomerase I inhibitor, camptothecin. Camptothecin has been shown to initiate apoptotic death of embryonic cortical neurons in a manner dependent on Bax and the conserved mitochondrial pathway of death including cytochrome *c* release and apoptosome activation (Morris and Geller 1996). This conserved mitochondrial pathway of death is only active when both the cell cycle and p53 upstream regulatory pathways are activated. Inhibition of either pathway blocks the conserved mitochondrial death signal (Morris *et al.* 2001; Sedarous *et al.* 2003). Based upon our initial gene array screen, we focused on the role of Pim-1 as an additional regulator of DNA damage induced neuronal death. We focused on (i) whether Pim-1 activity was changed (ii) whether Pim-1 participates functionally in death and (iii) how Pim-1 is regulated and the mechanism(s) by which it signals death.

Pim-1 is up-regulated after exposure to DNA damage and a pro-death regulator
We first examined whether Pim-1 levels change in embryonic cortical neurons after DNA damage. Cortical neurons derived from E15 mouse brain were exposed camptothecin (10 μM) for various time periods and were analyzed for Pim-1 mRNA and protein by RT-PCR and western immunoblotting, respectively. Pim-1 transcripts were detectably up-regulated as early as 2 h of camptothecin exposure and peaked by 4-h treatment (Fig. 1a). As shown in Fig. 1(b), Pim-1 signals were quantified by densitometry and normalized to the corresponding S12 control for each sample. This quantitation showed that camptothecin treatment increased Pim-1 mRNA levels by 3.5-, 5.3- and 3-fold at the 2-h, 4-h and 8-h time points, respectively. To determine whether camptothecin-induced increase in Pim-1 mRNA level was translated into an increase in Pim-1 protein level, we performed immunoblot analyses using anti-Pim-1 antibody. The murine Pim-1 is expressed as two isoforms: a 33-kDa protein (p33 Pim-1) and a 44-kDa protein (p44 Pim-1) (Saris *et al.* 1991). 44-kDa Pim-1 is the product of the same gene from which an upstream alternative translation initiation site was used during translation (Saris *et al.* 1991) and has comparable kinase activity as 33-kDa protein (Lilly *et al.* 1999). The Pim-1 antibody utilized recognizes both isoforms by western blot analyses which are not detected in Pim-1 deficient neurons (Fig. 1e). Figure 1(c) and (d) show that camptothecin treatment induced a significant increase in the level of both Pim-1 isoforms in cortical neurons. Pim-1 p44 isoform is more readily detectable than the p33 form under basal conditions. Induction of Pim-1 protein was detected as early as 1 h (data not shown). Pim-1 protein

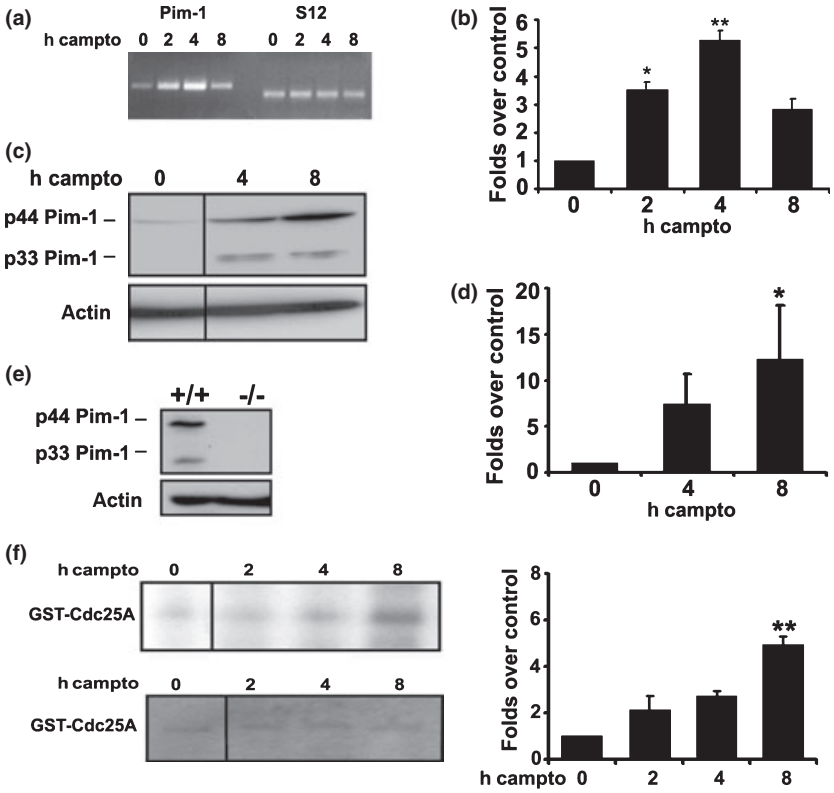

Fig. 1 Pim-1 activation in response to DNA damage. (a) Pim-1 mRNA increases early in response to camptothecin treatment. Total RNA was extracted from cortical neurons at the indicated times after camptothecin treatment (10 μM) and analyzed for Pim-1 mRNA expression using RT-PCR. S12 levels are shown as a control for equal input. (b) Pim-1 and S12 bands from three independent experiments were analyzed by densitometry using NIH ImageJ. Data for Pim-1 expression were normalized to S12 and expressed relative to the zero time point. Each point represents the mean ± SEM. Significance comparisons with untreated controls. *$p < 0.05$, **$p < 0.01$. (c) Pim-1 protein level increases with camptothecin treatment. Western blot showing relative Pim-1 levels from the whole cell lysates of cortical neurons following camptothecin (10 μM) treatment for the indicated times. An antibody that recognizes both the 44- and 33-kDa forms of Pim-1 was used. Actin is provided as loading control. (d) Densitometric analysis of western immunoblots. Data are the mean ± SEM and are expressed relative to the amount of Pim-1 at time 0. Significance comparisons with untreated controls. *$p < 0.05$. (e) The specificity of the Pim-1 antibody is demonstrated by using cortical cultures obtained from Pim-1-deficient and wild-type littermate controls. **(f)** Pim-1 kinase activity is rapidly increased in response to DNA damage. Pim-1 activity was calculated by measuring the incorporation of [γ-^{32}P]ATP onto GST-Cdc25A substrate and subtracting the control background value. Left panel, an example of autoradiogram showing the incorporation of [γ-^{32}P]ATP onto Cdc25A. Right panel, the kinase activity was quantified by densitometry using NIH ImageJ. Data represent the mean + SEM from three independent experiments. Significance comparisons with untreated controls. *$p < 0.05$. Bottom panel, SDS–polyacrylamide gel electrophoresis gel showing the substrate GST-Cdc25A.

levels were highest at the 8-h time point examined. This is in contrast to the peak of Pim-1 message observed at 4 h. This indicates that the stability of message and protein may differ or that there may be additional modes of Pim-1 regulation. Taken together, these data indicate that the early induction of Pim-1 RNA levels correlates well with increases in protein expression. To determine whether the elevation in Pim-1 levels is accompanied by an increase in kinase activity, we performed an *in vitro* kinase assay of Pim-1 immunoprecipitated from cortical neurons treated with camptothecin for various time periods utilizing Cdc25A as substrate. As shown in Fig. 1(f), activity of Pim-1 was increased throughout the time course of camptothecin treatment up to 8 h when compared to basal untreated controls.

We next asked whether the observed induction of Pim-1 was general to all inducers of apoptosis. To test this, we used the general apoptotic initiator and kinase inhibitor, staurosporine. Staurosporine can induce neuronal apoptosis at relatively low concentration (1 μM) (Koh *et al.* 1995). As shown in Fig. 2(a–c), neither Pim-1 message, protein level nor kinase activity changed upon staurosporine treatment. This indicates that Pim-1 involvement is not general to all apoptotic contexts.

The above evidence indicates that Pim-1 activity and levels is increased following camptothecin exposure. We

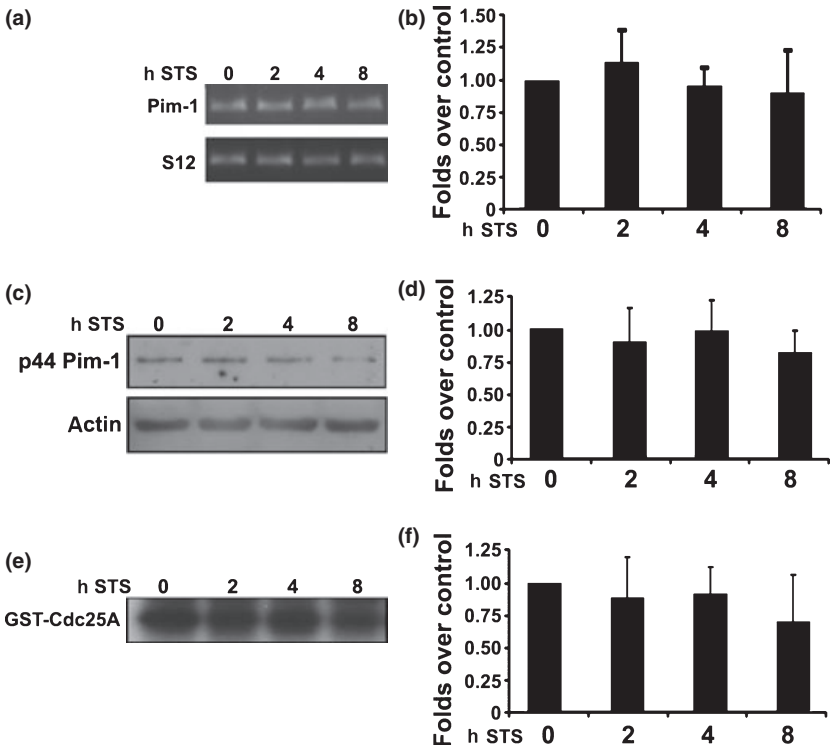

Fig. 2 Pim-1 is not activated in response to staurosporine. (a) Pim-1 mRNA does not increase in response to staurosporine (STS) treatment. Total RNA was extracted from cortical neurons at the indicated times after staurosporine treatment (1 μM) and analyzed for Pim-1 mRNA expression using RT-PCR. S12 levels are shown as a control for equal input. (b) Pim-1 and S12 bands from three independent experiments were analyzed by densitometry using ImageJ. Data for Pim-1 expression were normalized to S12 and expressed relative to the zero time point. Each point represents the mean ± SEM. **(c)** Pim-1 protein level does not change with staurosporine treatment. Western blot showing relative Pim-1 levels from the whole cell lysates of cortical neurons following staurosporine (1 μM) treatment for the indicated times. An antibody that recognizes both the 44- and 33-kDa forms of Pim-1 was used. Actin is provided as loading control. (d) Densitometric analysis of western immunoblots. Data are the mean ± SEM and are expressed relative to the amount of Pim-1 at time 0. (e) Pim-1 kinase activity does not change significantly in response to staurosporine treatment. Pim-1 activity was calculated by measuring the incorporation of [γ-^{32}P]ATP onto GST-Cdc25A substrate and subtracting the control background value. (f) The kinase activity was quantified by densitometry using NIH ImageJ. Data represent the mean + SEM from three independent experiments.

next examined the function of Pim-1 as it relates to neuronal death in this system. To do this, neurons were cultured from Pim-1 deficient embryos or heterozygous or wild-type littermate controls. As shown in Fig. 3(a), Pim-1-deficient neurons were significantly resistant to camptothecin-induced death when compared with wide-type controls (83% survival in knockout vs. 57% in wild-type controls at 8 h and 34% survival in wild-type vs. 58% knockout at 12 h). This finding supports a pro-apoptotic role for acute Pim-1 activation in response to DNA damage. We also performed Pim-1 over-expression experiment to test whether exogenous Pim-1 expression induces neuronal death. As shown in Fig. 3(b), Pim-1 expression alone is insufficient to induce death. It is also important to note that protection afforded by Pim-1 deficiency is not complete and transient. This suggests the participation of other death signals which act independently of Pim-1 (see Discussion below).

Cdc25A as downstream effector of Pim-1 in camptothecin-induced neuronal death

The above results suggest that Pim-1 is a pro-death factor in our paradigm of neuronal death. We next examined the mechanism(s) by which Pim-1 modulates death. Pim-1 has been shown to act on a number of substrates involved in cell cycle regulation such as Cdc25A, Cdc25C, p21 and C-TAK1 (Mochizuki *et al.* 1999; Wang *et al.* 2002; Bachmann *et al.* 2004, 2006). Relevant to the present work, however, is the report that Pim-1 can potentially regulate cell cycle through direct phosphorylation and resultant activation of Cdc25A phosphatase in proliferating cells (Mochizuki *et al.* 1999). To examine this potential mechanism, we first tested whether there is a physical interaction between Pim-1 and Cdc25A in post-mitotic neurons. We carried out immunoprecipitation (IP)-western blot assay using mouse brain exacts. Cdc25A was immunoprecipitated using an anti-Cdc25A antibody and co-immunoprecipitated proteins were examined for the

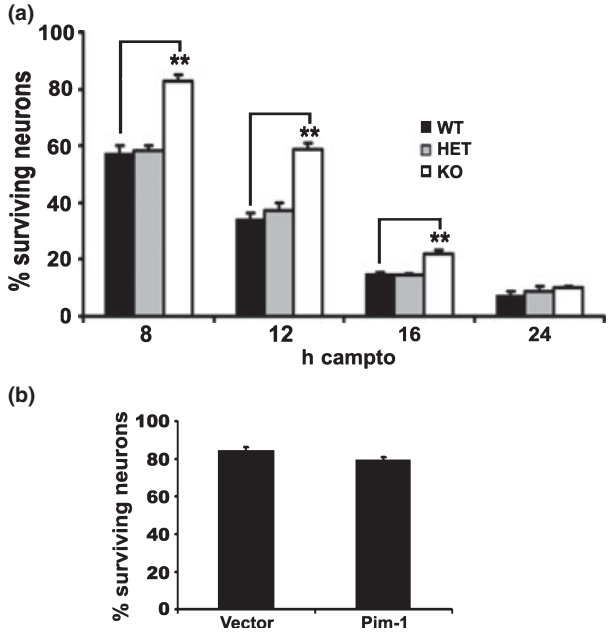

Fig. 3 (a) Pim-1 deficiency is protective against camptothecin-induced neuronal death. Cortical neurons were obtained from E15 embryos derived from a double heterozygote breeding of Pim-1 mice. Cultures were exposed to camptothecin (10 μM) for 8, 12, 16 and 24 h and cell viability was analyzed by nuclear counts. $^{**}p < 0.01$ compared with control. Each data point is the mean ± SEM from at least three independent embryos. (b) Pim-1 over-expression does not induce significant cell death. Cultured cortical neurons were co-transfected with pEGFP and the indicated constructs. 24 h after transfection, cells were fixed and stained with Hoechst. GFP-positive neurons were scored for survival by evaluating nuclear integrity.

presence of Pim-1 by western blot analyses. In Fig. 4(a), we show that IP with Cdc25A antibody but not an IgG control antibody pulls down Pim-1. The reciprocal experiment was also performed where IP was performed using Pim-1 antibody followed by probing for Cdc25A. Consistent with the above results, an interaction between Pim-1 and Cdc25A was also observed (Fig. 4b). This indicates that these two proteins can interact endogenously in brain. To further support the interaction of Pim-1 and Cdc25A, we performed co-immunoprecipitation assay using cultured neurons. As shown in Fig. 4(c), endogenous Cdc25A was detected by western blot analyses when Pim-1 was immunoprecipitated utilizing Pim-1 antibody. Finally, we examined whether Cdc25A and Pim-1 would colabel upon immunofluorescence analyses in cortical neurons (Fig. 4d). Upon camptothecin treatment there was a noticeable increase in Pim-1 staining as predicted by our previous biochemical experiments. There was also significant overlap between the Pim-1 and Cdc25A signals supporting the observed physical interaction between the two proteins as detected by IP analyses.

Next, to test the hypothesis that Pim-1 modulates neuronal death by enhancing Cdc25A activity in this paradigm, we determined whether/how Pim-1 deficiency would affect Cdc25A activation previously observed following DNA damage (Zhang *et al.* 2006). To do this, Cdc25A activity was measured from Pim-1 deficient or littermate wild-type neuronal cultures treated with camptothecin utilizing 4-nitrophenol phosphate as a substrate. As shown in Fig. 4(e), Cdc25A phosphatase activity was increased in wild-type neuronal cultures upon camptothecin exposure, consistent with our previously reported results. However, this increase was blunted in Pim-1 deficient cultures. Pim-1 expression alone was not sufficient to Cdc25a activation (Fig. S1). Cyclin D1-associated kinase activity is also increased in wild-type neurons following DNA damage in a Cdc25A dependent fashion. If Pim-1 truly affects Cdc25A activity, we would expect that Cyclin-D1 associated kinase activity would also decrease. As shown in Fig. 4(f), the induction of Cyclin D1 activity was also abolished in Pim-1 deficient neurons. These findings indicate that Pim-1 mediates neuronal death upstream of Cdc25A and Cdk/Rb/E2F.

NF-κB as activator for Pim-1 up-regulation at transcriptional level

Thus far we have shown that Pim-1 functions to regulate Cdc25A in this DNA damage paradigm. Next, we investigated how DNA damage up-regulates Pim-1 expression. It has been demonstrated that Pim-1 is induced by transcription factor NF-κB in CD40 signaling in B lymphocytes (Zhu *et al.* 2002). This is consistent with the observation that the Pim-1 promotor (both human and mouse) contains several putative NF-κB binding sites situated closely together (Fig. 5a). Accordingly, we first examined whether NF-κB members can potentially bind these sites. We designed primer sequences (using DNASTAR software) close to the NfkB binding region and performed chromatin immunoprecipitation (ChIP) analyses for p65 and c-Rel (Fig. 5b and c). Our results demonstrated that both mouse and human Pim-1 promoter contains p65. The human Pim-1 promoter also binds c-Rel which was not detected with the mouse promoter (data not shown). These experiments indicate that NF-κB subunits can bind to the Pim-1 promoter. Supporting the functional relevance of this binding, we also tested whether association of p65 to Pim-1 promoter is augmented in response to DNA damage. As shown in Fig. 5(d), promoter binding ability of p65 increases with camptothecin treatment. Next we examined for the effects of p65 expression on a luciferase reporter construct driven by the Pim-1 promoter containing the NF-κB sites. As shown in Fig. 5(e), p65 expression significantly increases the Pim-1-promoter driven luciferase signal. The above evidence combined with our previous report that NF-κB modulates DNA damage response as a pro-death factor in this death model (Aleyasin *et al.* 2004) prompted us to address the possibility that camptothecin-induced Pim-1 expression might be directly controlled by NF-κB. To this end, we employed two different

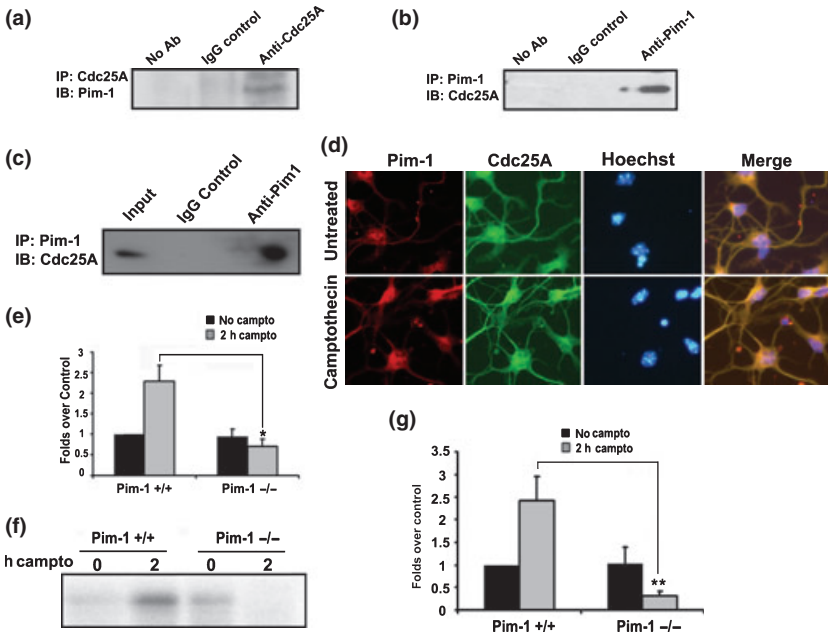

Fig. 4 Pim-1 regulates Cdc25A in response to DNA damage. (a and b) *In vivo* association of Pim-1 and Cdc25A. IP-western blot analysis of mouse brain extracts immunoprecipitated with anti-Cdc25A (or anti-Pim-1) antibody. Immunoprecipitates were run on SDS–polyacrylamide gel electrophoresis followed by western blot analysis with anti-Pim-1 (or anti-Cdc25A) antibody. Parallel IP-western blot analysis using no antibody or non-specific IgG antibody were performed as controls. (c) Association of Pim-1 and Cdc25A in cultured neurons. Cultured cortical neurons were treated with camptothecin for 8 h. Pim-1 protein was immunoprecipitated by an anti-Pim-1 antibody and western blot was performed using an anti-Cdc25A antibody. (d) Pim-1 co-localized with Cdc25A in cortical neurons. Cortical cultures were treated with or without camptothecin for 4 h and subjected to Pim-1 and Cdc25A immunostaining. Nuclei were stained with Hoechst. (e) Pim-1 deficiency attenuates camptothecin-induced Cdc25A phosphatase activity. Cortical neurons from Pim-1+/+ and Pim-1−/− littermates were treated with or without camptothecin for 2 h. Cdc25A was immunoprecipitated from whole-cell lysate of neurons and subjected to phosphatase assay using pNPP as substrate. Data represent the mean ± SEM from four independent experiments. $^{*}p < 0.05$. (f) Pim-1 deficiency attenuates camptothecin-induced Cyclin D1-associated kinase activity in cortical neurons. Cdk4/6 were immunoprecipitated from whole-cell lysate of Pim-1+/+ and Pim-1−/− neurons treated with or without camptothecin for 2 h and subjected to kinase assay using pRb as substrate. (g) Densitometric analysis of Cyclin D1-assciated kinase activity. The kinase activity was quantified by measuring the incorporation of [γ-^{32}P]ATP onto pRb substrate and subtracting the wild-type control background value. Data represent the mean ± SEM from five independent experiments. $^{**}p < 0.01$.

approaches to inhibit NF-κB activation and observe whether camptothecin-evoked up-regulation of Pim-1 is affected. First, we used several different pharmacological inhibitors to block NF-κB activity via different mechanisms: (i) CAPE inhibits nuclear translocation of NF-κB possibly via antioxidant effects (Natarajan *et al.* 1996); (ii) Helenalin blocks DNA binding of NF-κB by specific and irreversible alkylation of the p65 subunit (Lyss *et al.* 1997, 1998); (iii) BAY 11-7082 inhibits IκBα phosphorylation and degradation thereby results in the inactivation of NF-κB (Pierce *et al.* 1997). We treated cortical neurons with camptothecin (10 μM) alone or co-treated with CAPE, BAY 11-7082 and Helenalin for the indicated time periods. Total RNA and protein were harvested for RT-PCR and western blot analysis, respectively. As shown in Fig. 6(a), RT-PCR demonstrated that co-treatment of NF-κB inhibitors significantly decreases Pim-1 signals at 4 h after camptothecin treatment. Similarly, co-treatment of NF-κB inhibitors robustly decreases Pim-1 protein level at 4 h and 8 h after camptothecin treatment (Fig. 6b). Importantly, Helenalin and CAPE also markedly decreased camptothecin induced kinase activity (Fig. 6c).

Because of potential specificity issues related to pharmacological inhibitors, we next examined the functional link of NF-κB and Pim-1 using siRNA-mediated knockdown approach. In this regard, we transfected neurons with two independent NF-κB-specific siRNA oligonucleotide reagents and treated with camptothecin for the indicated times. RNA and protein levels of Pim-1 were examined by RT-PCR and western blot analysis respectively. Figures 7a,b and S2a,b show that down-regulation of NF-κB significantly decreases Pim-1 induction at both RNA and protein levels. Also, siRNA-mediated down-regulation of p65 leads to significantly decreased Pim-1 kinase activity induced by DNA damage (Figs 7c and S2c). These results are consistent with the observations that NF-κB, as a pro-apoptotic factor, is

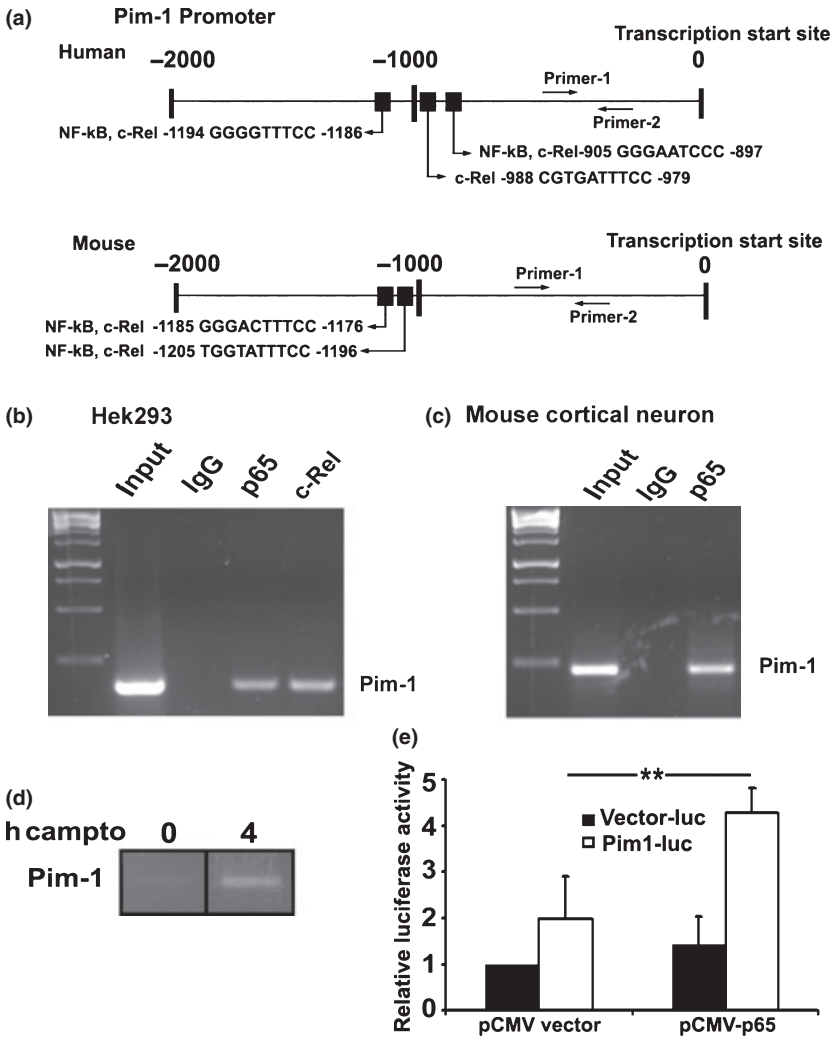

Fig. 5 NF-κB interacts with Pim-1 promoter in both mouse and human cells. (a) NF-κB binding sites in Pim-1 promoter region as identified through the TFSEARCH server (http://www.cbrc.jp/research/db/TFSEARCH.html). (b) ChIP assay to show p65 and c-Rel subunits bind to Pim-1 promoter in HEK293 cells. (c) ChIP assay to show p65 subunit binds to Pim-1 promoter in mouse cortical neurons. (d) Promoter binding ability of p65 increases with camptothecin treatment. Cultured neurons were treated with camptothecin for 4 h and ChIP assay was performed as described above. (e) p65/Pim-1 promoter luciferase assay. Luciferase vector (vector-luc) and Pim-1 promoter luciferase construct (Pim-1-luc) were co-transfected with a pCMV control vector or pCMV-p65 plasmid as well as CMV-β-galactosidase plasmid using Lipofactamine in HEK293 cells. 24 h after transfection, cell lysates were processed for both luciferase and β-galactosidase assay. Data are presented as normalized luciferase activity (luciferase/β-galactosidase). $^{**}p < 0.05$.

activated early (as early as 2 h after camptothecin treatment) in response to DNA damage (Aleyasin *et al.* 2004), and support an important role of NF-κB as upstream activator of cell cycle pathway via Pim-1 in this death model.

p53 is not a downstream effector of Pim-1 in camptothecin-induced neuronal death, and vice versa

As we have previously shown that NF-κB mediates death through induction of p53 following DNA damage (Aleyasin *et al.* 2004), we asked whether NF-κB regulates death and p53 induction via Pim-1 up-regulation in this death paradigm. Previously we have shown that p53 is induced after camptothecin exposure, accompanied by an increase in p53-responsive genes (Morris *et al.* 2001; Cregan *et al.* 2004). The induction of the Bcl-2 family members Noxa and Puma is known to be dependent on p53 (Oda *et al.* 2000; Yu *et al.* 2001). Accordingly, we examined whether Pim-1 acts as an upstream regulator of p53 pathway. As shown in Fig. 8(a), the induction of Noxa and Puma in Pim-1 wild-type neurons after camptothecin treatment, as determined by RT-PCR, is not reduced in Pim-1-deficienct neurons. These results indicate that Pim-1 does not act upstream of p53 in this death model. Next we addressed the question whether p53 regulates Pim-1 up-regulation in this death paradigm. To this end, we examined Pim-1 induction in response to DNA damage in p53 deficient neurons. As shown in Fig. 8(b), p53 deficiency did not affect camptothecin-induced Pim-1 up-regulation.

Discussion

DNA damage triggers a complex series of biochemical and molecular mechanisms which eventually results in rapid neuronal cell death. These intricate processes lead to activation of at least three signaling pathways: (i) a cell cycle pathway which involves Cdc25A phosphatase, Cyclin D1-associated kinases, pRb family members, and E2F transcription factors (Park *et al.* 1997); (ii) a p53 pathway

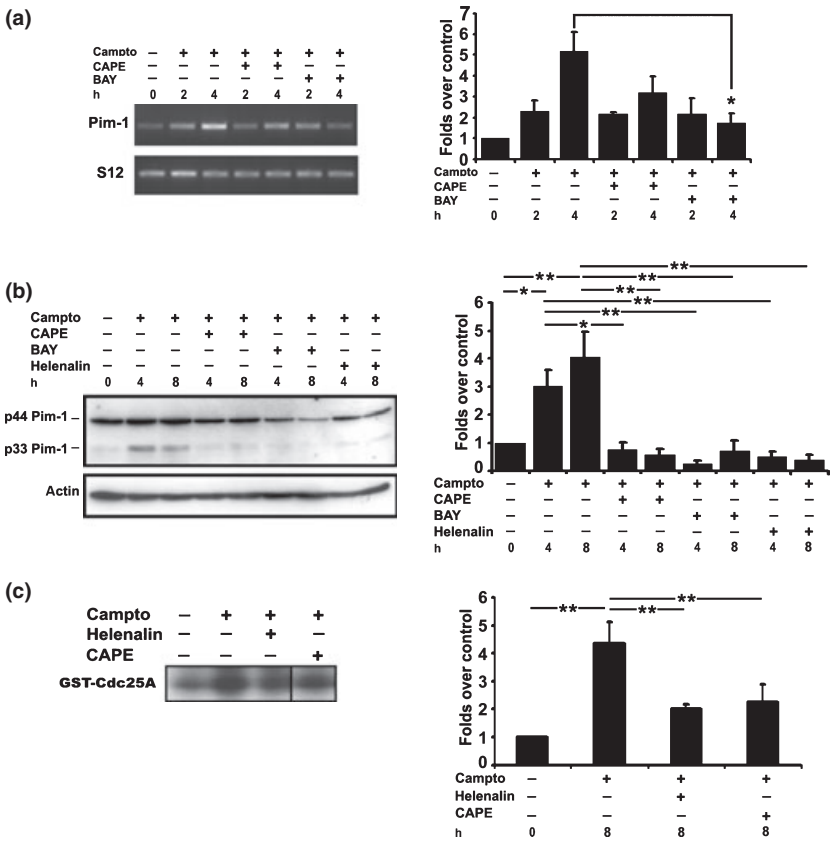

Fig. 6 NF-κB inhibition blocks DNA damage-induced up-regulation of Pim-1. (a) Cortical neurons were treated with camptothecin (10 μM) alone or camptothecin plus CAPE (5 μM) and BAY 11-7082 (5 μM) for 2 and 4 h. Total RNA was extracted and Pim-1 transcripts were analyzed by RT-PCR. S12 was analyzed as a control (left panel). Densitometric analysis of RT-PCR was performed from three independent experiments (right panel). Data represent the mean ± SEM from three independent experiments. *$p < 0.05$. (b) Cortical neurons were treated with camptothecin (10 μM) alone or camptothecin plus CAPE (5 μM), BAY 11-7082 (5 μM) and Helenalin (5 μM) for 4 and 8 h. Whole cell extracts were separated by SDS–polyacrylamide gel electrophoresis and then analyzed by immunoblotting with anti-Pim-1 antibody. Actin is provided as loading control (left panel). Densitometric analysis of three independent experiments was performed (right panel). Data represent the mean ± SEM from three independent experiments. *$p < 0.05$, **$p < 0.01$. (c) NF-κB inhibition blocks Pim-1 kinase activity induced by DNA damage. Cortical neurons were treated with camptothecin (10 μM) alone or camptothecin plus Helenalin (5 μM) or CAPE (5 μM) for 8 h. Pim-1 were immunoprecipitated and subjected to kinase assay using Cdc25A as substrate (left panel). Densitometric analysis of three independent experiments was performed (right panel). Data represent the mean ± SEM from three independent experiments. *$p < 0.05$.

which is regulated by NF-κB and ataxia telangiectasia mutated (ATM) kinase (Aleyasin *et al.* 2004); (Keramaris *et al.* 2003) and (iii) a c-*Jun* N-terminal kinases (JNKs) pathway (Ghahremani *et al.* 2002). The coordination of these and likely other signal transducers is necessary to activate the conserved mitochondrial pathway of death. However, upon DNA damage, the manner by which these cellular events are initiated, regulated and interact to promote neuronal death is not fully understood. The purpose of the present study is to further our understanding of the molecular mechanisms involved in these processes, particularly in regards to cell cycle activation. Presently, the significance of our findings are as follows: (i) we provide critical evidence of how abnormal cell cycle activation is promoted in neurons by Pim-1. This evidence is crucial if we are to understand the unique phenomenon of cell cycle in neuronal death. (ii) We demonstrate that Pim-1 plays a prodeath role in neurons exposed to DNA damage, contrary to the prosurvival properties attributed to this kinase in dividing cells. (iii) We delineated the mechanism by which Pim-1 is activated (NF-κB) and its downstream target linking it to the cell cycle in neurons (Cdc25A).

Pim-1 and cell cycle regulation in neuronal death

In this study, we report that Pim-1 mediates DNA-damage induced neuronal death through enhancing Cdc25A activity and that this is critical for the inappropriate activation of the cell cycle signals in neurons. The data supporting this model is as follows: (i) Pim-1 levels and activity are induced following DNA damage. (ii) Pim-1 activity is important for

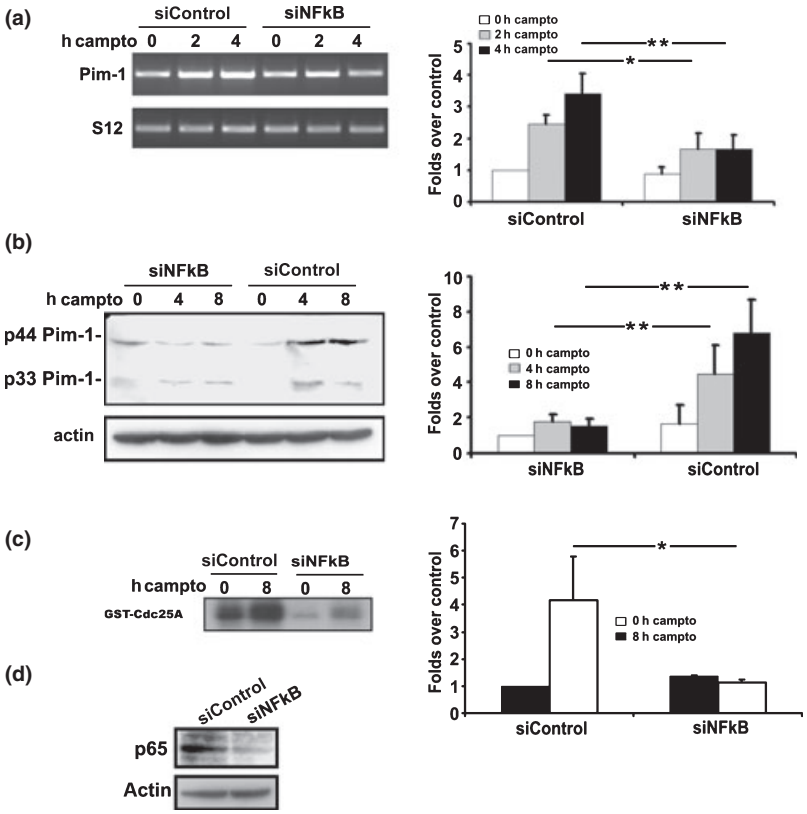

Fig. 7 NF-κB knockdown blocks DNA damage-induced up-regulation of Pim-1. (a) Cortical neurons were transfected with a control siRNA (siControl) or NF-κB siRNA oligonucleotides (siNF-κB) for 24 h and then treated with camptothecin for 2 and 4 h as indicated. Pim-1 transcripts were analyzed by RT-PCR (left panel). Densitometric analysis of three independent experiments are presented as fold increase compared with untreated groups transfected with control siRNA (right panel). Data represent the mean ± SEM. $*p < 0.05$, $**p < 0.01$. (b) Cortical neurons were transfected as above mentioned and whole-cell lysates prepared from transfected cortical neurons were subjected to western blot analysis using anti-Pim-1 antibody. Actin is provided as loading control (left panel). Densitometric analysis of western immunoblots was performed as mention above (right panel). (c) Cortical neurons were transfected as above mentioned and then treated with camptothecin for 8 h as indicated. Pim-1 were immunoprecipitated and subjected to kinase assay using Cdc25A as substrate (left panel). Densitometric analysis was performed as mention above (right panel). (d) Western blot analyses showing down-regulation of NF-κB p65 subunit in cells treated with NF-κB siRNA compared with control siRNA. Actin is provided as loading control.

death as its deficiency delays death. (iii) Pim-1 deficiency also blocks Cdc25A activity, an activity that we have previously shown to be important for activation of Cyclin D1-associated kinase activity following DNA damage in neurons and consequent death. (iv) Pim-1 deficiency also blocks Cdk4/6 activity consistent with its importance in regulation of Cdc25A activity. Previously we have also reported that Cdc25A activity is also regulated by the checkpoint kinase Chk1. In this case, loss of basal Chk1 kinase activity contributes to increased Cdc25A activity. Accordingly, taken together with our current data, we suggest a model by which Cdc25A regulation is controlled by at least two different pathways, one involving the loss of Chk1 activity, and the other regulated by an increase in Pim-1 kinase activity. This is consistent with our observation that over-expression of Pim-1 alone does not lead to neuronal death or increased Cdc25A activity (Figs 3b and S1). This dual control of Cdc25A activation makes sense in light of the need to ensure that activation of the critical Cdk death signal not occur unless warranted. This 'dual key' theme in death regulation is common and likely occurs at multiple levels to prevent accidental death. For example, we have previously shown that activation of the conserved mitochondrial death machinery is also regulated by both p53 and Cdk activation (Morris *et al.* 2001). In the case of Pim-1, this dual regulation is likely even more critical as Pim-1 may have normal neuronal function in limited contexts such as with consolidation of long-term potentiation (Konietzko *et al.* 1999).

While we have provided evidence for a Pim-1/Cdc25A relevant pathway, we do not rule out the possibility that Pim-1 may also act to regulate other factors. Indeed, even in regards to cell cycle regulation, Pim-1 can modulate a number of substrates. This includes the Cdk inhibitors, p21

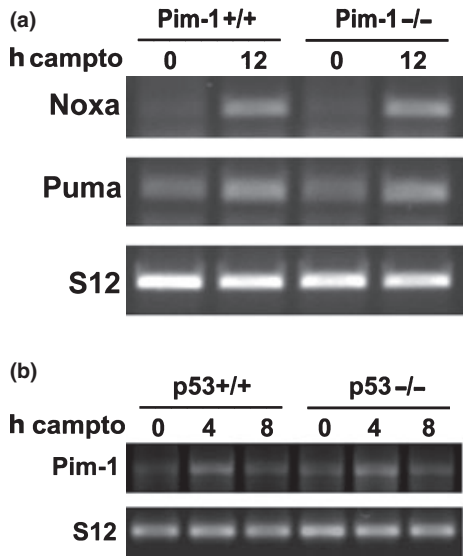

Fig. 8 (a) Pim-1 deficiency does not inhibit the p53-inducible genes Noxa and Puma. Cortical neurons from Pim-1+/+ and Pim-1–/– mice were treated with and without camptothecin for 12 h. Noxa and Puma levels were then analyzed by RT-PCR as described in Material and Methods. S12 was also analyzed as a control for equal input. (b) p53 deficiency does not affect Pim-1 induction in response to camptothecin treatment. Cortical neurons from p53+/+ and p53–/– mice were treated with and without camptothecin for 4 and 8 h. S12 was also shown as a control for equal input.

involved in G1/S progression and Cdc25C, a Cdc25 family member associated with G2/M progression (Wang *et al.* 2002; Bachmann *et al.* 2006). In regards to the former, Pim-1-mediated phosphorylation of p21 leads to its cytoplasmic sequestration, at least in proliferating cells (Wang *et al.* 2002). However, we did not observe any translocation of p21 in Pim-1 deficient neurons when compared to wild-type controls either basally or in the presence of DNA damage (unpublished data). Cdc25C directs dephosphorylation of Cyclin B-bound Cdc2 and triggers entry into mitosis during G2/M transition (Strausfeld *et al.* 1991). Interestingly, Cdc2 is implicated in multiple neuronal death models including K deprivation and in response to DNA damaging agent (Konishi *et al.* 2002; Konishi and Bonni 2003; Ueno *et al.* 2006). Whether this cell cycle Cdk member as well as a potential corresponding Pim-1/Cdc25C pathway of regulation is important in neuronal death will be interesting to explore more carefully.

Finally, it is important to reiterate that death regulation likely involves numerous pathways acting in concert to execute the life-death decision. In this regard, Pim-1 is only one player participating in this balancing act. This is evidenced by the fact that Pim-1 deficiency only delays neuronal death and does not completely prevent it. It is also possible that activation of the other two Pim family members: Pim-2 and Pim-3, which have similar activity and overlapping functions as Pim-1 (Bachmann and Moroy 2005; Bullock *et al.* 2005), may compensate for the loss of Pim-1 in this death model. However, it is also equally likely that there are other death signals which act independently of Pim-1 regulated pathways to promote death. This includes p53 which is activated independently of abnormal cell cycle activation (Morris *et al.* 2001).

Regulation of Pim-1 upon DNA damage

What regulates the increase in Pim-1 levels following DNA damage? We had previously shown that NF-κB plays a dual role in regulating neuronal loss where basal NF-κB activity is critical for survival while acute NF-κB activation signals death through induction of p53 message (Aleyasin *et al.* 2004). It is important to point out that NF-κB, however, is not the only regulator of p53. For example, post-translational modification of p53 is also regulated by upstream kinases such as ATM (Canman *et al.* 1998), which presumably regulates the stability of the p53 protein. p53 is also regulated by calpain activation in a manner which is not clear yet (Sedarous *et al.* 2003). Interestingly, we now show that NF-κB is also critical for induction of Pim-1 message/protein which in turn is critical for Cdk activation. This evidence suggests that NF-κB promotes the up-regulation of at least two pathways critical for DNA damage-induced death, one involving p53 and the other Cdk activation. This work is interesting in light of our previous findings that inhibition of Cdks did not affect p53 activation and that conversely, p53 deficiency did not affect DNA damage-induced pRb phosphorylation (Morris *et al.* 2001). We interpreted these observations to mean, at the time, that Cdks and p53 were on parallel pathways. However, our new data suggest that the signaling picture is a bit more complex and that there is some degree of cross-talk upstream of Cdks and p53. Finally, while our evidence suggests that regulation of Pim-1 levels is important, it does not rule out the potential importance of post-translational modification of Pim-1. For example, protein phosphatase 2A (PP2A), a serine/threonine phosphatase, which is reported to associate with Pim-1 *in vivo*, dephosphorylates Pim-1 and decreases its kinase activity (Losman *et al.* 2003; Ma *et al.* 2007). Interestingly, a recent study shows that PP2A appears to be a pro-survival factor during neuronal death and its phosphatase activity is significantly decreased following cerebral hypoxia (Truttmann *et al.* 2004). Whether or not PP2A is critical for Pim-1 regulation in neuronal death will be of interest for further study.

Relevance of this pathway in injury and disease

The potential importance of the NF-κB/Pim-1/Cdc25A/Cdk pathway in a wider context than that presented here, is potentially intriguing. Just as one example, many of the players described here have also been implicated in ischemic injury. There is now increasing evidence in multiple stroke models that cell cycle Cdks are activated and required for

death (Osuga *et al.* 2000; Katchanov *et al.* 2001; Rashidian *et al.* 2005, 2007). Our previous report has shown that inhibition of Cdk4, and its activator Cyclin D1, plays critical roles in delayed death component of ischemic/hypoxic stress by regulating the pRb protein (Rashidian *et al.* 2005). Although the potential role of the cell cycle regulators in neuronal death has been hypothesized, little has been done concerning how cell-cycle Cdks are activated, particularly in an *in vivo* context. Interestingly, NF-κB is activated following stroke and appears to have both protective and deleterious roles similar to our DNA damage models *in vitro* (Schneider *et al.* 1999). Finally, Cdc25A phosphatase activity increases in cerebellar granule neurons upon hypoxia/reoxygenation and one of the pharmaceutical inhibitors for Cdc25A, NSC95397 protects these neurons from hypoxia-induced death (unpublished data). Whether or not these signals are linked and regulated by Pim-1 in a similar fashion in injury models such as stroke or other degenerative diseases where Cdks appear to be activated will be an important question for future research.

In conclusion, past and present data present a growing model by which a complex array of signals regulate three major pathways of death cell cycle Cdks, p53, and the JNKs (see Fig. 9). In the Cdk branch of signals, Pim-1 activation and loss of Chk1 activity both contribute to Cdc25A activation and consequent increase in Cdk4/6 activity. Pim-1 activity is regulated, at least in part by an increase in levels regulated by NF-κB. NF-κB also works in conjunction with other regulators such as ATM and calpains to regulate p53 activation. Cdks act on the tumor suppressor pRb family members which in turn regulate downstream effectors such as B-myb and C-myb (Liu *et al.* 2004). Cdks also activate the PP2A inhibitor I2PP2A protein (Qu *et al.* 2007) and the transcription co-activator Cited2 which acts in turn to regulate a peroxisome proliferator-activated receptor-γ mediated pathway of death (65). p53, once activated regulates a series of pro-death molecules such as Puma and Apaf (Fortin *et al.* 2001; Cregan *et al.* 2004; Uo *et al.* 2007). There is also at least a third pathway involving the stress activated JNKs (Ghahremani *et al.* 2002). This pathway appears to be regulated by upstream Cdc42 and plenty of SH3 domains/JNK interacting proteins as well as Cdks (Ghahremani *et al.* 2002; Kukekov *et al.* 2006). These three pathways coordinate ultimately, in ways which are not completely clear, to control the translocation and activation of Bax to the mitochondria, cytochrome *c* release and activation of downstream effector caspases. Accordingly, our present data delineate the regulatory steps of a critical pathway in DNA damage induced death provides insight into the understanding of mechanisms of cell cycle activation.

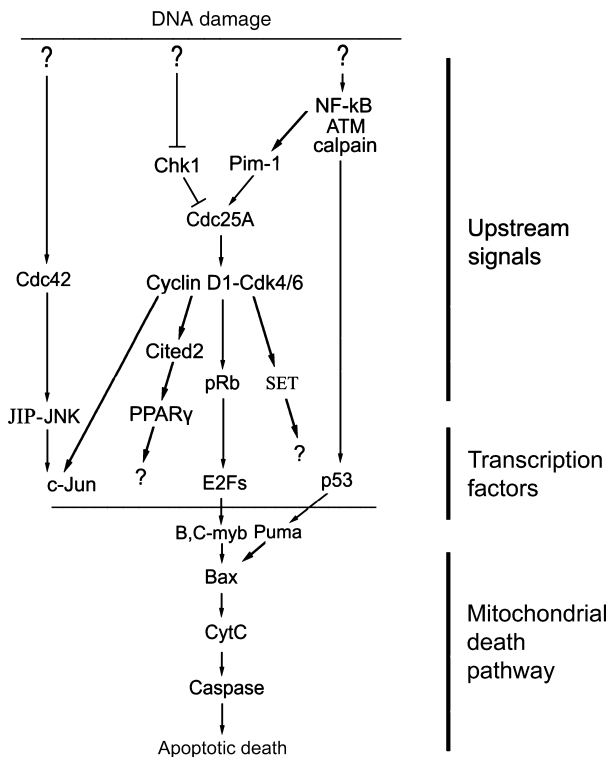

Fig. 9 Schematic model of the signaling pathways involved in DNA damage induced death.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Pim-1 over-expression does not significantly enhance Cdc25A activity.

Figure S2. NF-κB down-regulation blocks DNA damage-induced up-regulation of Pim-1.

Appendix S1. Supplementary Materials and Methods.

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Role of Cdk5-Mediated Phosphorylation of Prx2 in MPTP Toxicity and Parkinson's Disease

Dianbo Qu,[1] Juliet Rashidian,[1,3] Matthew P. Mount,[1,3] Hossein Aleyasin,[1,3] Mohammad Parsanejad,[1] Arman Lira,[1] Emdadul Haque,[1] Yi Zhang,[1] Steve Callaghan,[1] Mireille Daigle,[1] Maxime W.C. Rousseaux,[1] Ruth S. Slack,[1] Paul R. Albert,[1] Inez Vincent,[2] John M. Woulfe,[1] and David S. Park[1,*]
[1] Ottawa Health Research Institute, Neuroscience Group, University of Ottawa, Ottawa, K1H 8M5 ON, Canada
[2] Centre for Molecular Medicine and Therapeutics, University of British Columbia, 950 West 28th Avenue, Vancouver, V5A 4H4 BC, Canada
[3] These authors contributed equally to this work.

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SUMMARY

We reported previously that calpain-mediated Cdk5 activation is critical for mitochondrial toxin-induced dopaminergic death. Here, we report a target that mediates this loss. Prx2, an antioxidant enzyme, binds Cdk5/p35. Prx2 is phosphorylated at T89 in neurons treated with MPP^+ and/or MPTP in animals in a calpain/Cdk5/p35-dependent manner. This phosphorylation reduces Prx2 peroxidase activity. Consistent with this, $p35^{-/-}$ neurons show reduced oxidative stress upon MPP^+ treatment. Expression of Prx2 and Prx2T89A, but not the phosphorylation mimic Prx2T89E, protects cultured and adult neurons following mitochondrial insult. Finally, downregulation of Prx2 increases oxidative stress and sensitivity to MPP^+. We propose a mechanistic model by which mitochondrial toxin leads to calpain-mediated Cdk5 activation, reduced Prx2 activity, and decreased capacity to eliminate ROS. Importantly, increased Prx2 phosphorylation also occurs in nigral neurons from postmortem tissue from Parkinson's disease patients when compared to control, suggesting the relevance of this pathway in the human condition.

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder characterized by motor symptoms including tremor, muscle rigidity, paucity of voluntary movements, and postural instability (Hoehn and Yahr, 1967; Lang and Lozano, 1998). The pathological hallmarks of PD are the loss of dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNc) and formation of Lewy bodies (Braak et al., 2003). The pathogenic process in PD is not clearly understood. A small portion (less than 10%) of PD patients have familial forms of the disease, and several PD genes have been identified (Abou-Sleiman et al., 2006). However, the vast majority of patients have idiopathic forms of PD. Parkinsonism can be induced in humans by exposure to the mitochondrial complex 1 toxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). This DAergic toxin results in parkinsonism symptoms indistinguishable from those of Parkinson's disease (Burns et al., 1985; Langston et al., 1983). Experimentally, it induces specific loss of DAergic neurons in the SNc (Burns et al., 1983) and produces a profound reduction of striatal dopamine levels with little alteration in other catecholamine neurotransmitter systems (Jonsson et al., 1986). Degeneration is a consequence of conversion of MPTP by glia to its toxic metabolite MPP^+ followed by specific uptake by DAergic neurons and presumed targeting of the mitochondria (Lang and Lozano, 1998).

The cellular consequences of mitochondrial dysfunction as induced by agents such as MPTP are numerous and include poor calcium homeostasis and oxidative stress (Wang and Yuen, 1994). How this dysregulation occurs and the consequence/management of these stresses are not fully understood. Recently, and consistent with improper calcium management, we reported that calpains are activated and required for MPTP-induced death in adult mice in vivo (Crocker et al., 2003). Calpains are conserved cysteine proteases regulated by calcium and possessing diverse biological function (Sorimachi et al., 1997). This was consistent with earlier reports of elevated calpain levels in postmortem PD patients (Mouatt-Prigent et al., 1996). We also showed that calpains were more activated in PD patients than in control individuals (Crocker et al., 2003). Importantly, calpain inhibition not only limited DAergic loss but also improved animal behavior following toxin treatment (Crocker et al., 2003).

What are the possible downstream targets of calpain activation? We recently provided evidence that the activator of Cdk5, p35, may be critical (Smith et al., 2003, 2006). Cdk5, not thought to be central to the core cell-cycle machinery, has been implicated in brain function including neuronal development, neuritic outgrowth, and neurotransmitter (dopamine) signaling (Dhavan and Tsai, 2001). Cdk5 activity is regulated by its activating partners, p35 and p39 (Dhavan and Tsai, 2001). While important for brain

development, recent evidence has shown that inappropriate activation of the Cdk5/p35 signal may lead to neuronal death through pathogenic activation of calpains, which proteolytically cleave p35 to a more active p25 form (Dhavan and Tsai, 2001) at least in cultured systems. However, its functional role in adult degeneration in vivo as well as in PD was unknown. To this end, we found that Cdk5 plays an essential role in DAergic loss in vivo (Smith et al., 2003, 2006). For example, we observed that MPTP induced Cdk5 activation and that inhibition of such activity with DN Cdk5 expression, Cdk inhibitors, or p35 deficiency attenuated DAergic death and behavioral deficits associated with MPTP treatment (Smith et al., 2003, 2006). This observation is made more significant by observations of increased p35 in postmortem PD brains (Nakamura et al., 1997). It is also entirely consistent with our observations that calpains are activated, are required for death, and mediate p35 to p25 cleavage in the MPTP model of PD (Smith et al., 2003). In support of this, we showed that inhibition of calpain led to reduced p35 to p25 conversion and Cdk5 activation (Smith et al., 2006). This suggested to us a model by which deregulated calcium leads to calpain activation, inappropriate Cdk5 activity, and DAergic cell death (Smith et al., 2006). However, the mechanism(s) by which Cdk5 promotes DAergic loss is still unknown. To address this, we presently performed mass spectrometry-based interactomics to identify Cdk5-interacting proteins. We identified an intriguing target that has direct implications for the way in which cells handle oxidative stress, linking Cdk5 activity with oxidative stress, a common theme in PD.

Oxidative stress is thought to be a critical mediator of damage in PD (Przedborski, 2005). Reactive oxygen species (ROS) has been observed in the SNc of PD patients and animal models of PD (Przedborski, 2005). Importantly, neurons in general have high levels of ROS. Therefore, systems to handle such stress are of paramount importance. Peroxidases are a key in this management system. Three types of peroxidases, peroxiredoxins (Prxs), catalase, and glutathione peroxide (GPx), function to eliminate H_2O_2 in mammalian cells (Rhee et al., 2005). In mammalian cells, six isoforms of Prx were identified. Although Prxs as a family are relatively ubiquitous, there is some specificity in regards to cell type and subcellular localization. For instance, Prx1 is distributed in the cytoplasm of oligodendrocytes and microglia, while Prx2 is located in the cytoplasm of neurons, and Prx3 is localized in the mitochondria of neurons (Jin et al., 2005). Whether and how Prxs participate in neuronal damage as well as how they are regulated have yet to be examined.

Presently, we report that Prx2 directly associates with the Cdk5 kinase complex through p35. In addition, Cdk5 phosphorylates Prx2 at T89 resulting in reduction of Prx2 peroxidase activity and neuronal death in MPP^+-treated cells in vitro and the MPTP mouse model of PD in vivo. Prx2 activity is functionally relevant since modulation of Prx2 activity is protective. Importantly, $p35^{-/-}$ neurons have reduced ROS and improved survival consistent with the above findings. These findings provide a mechanistic link of how a mitochondrial damaging agent, through calpain-mediated Cdk5 activation and downregulation of an important antioxidant enzyme, can increase oxidative load leading ultimately to death.

RESULTS

Identification of Prx2 as a Cdk5-Interacting Protein

We have shown that Cdk5 plays an essential role in loss of DAergic neurons in the MPTP mouse model of PD (Smith et al., 2003, 2006). It has also been demonstrated that Cdk5/p35 exists as macromolecular complexes in brain extracts, implying that Cdk5 associates with different proteins and functions in different signaling pathways (Lee et al., 1996). To identify these complexes, we initially utilized bacterially expressed GST-p10, a C-terminal truncated form of p35 containing a 98 amino acid residue N terminus fused with GST, as a bait to isolate p35-interacting proteins from mouse brain extracts. The interacting proteins were eluted with 1 M NaCl from GST-immobilized GSH beads. The eluted proteins were visualized by Coomassie blue staining after separation by SDS-PAGE (Figure 1A). The visualized specific bands were subjected to protein identification by tandem mass spectrometry. One of three specifically isolated proteins was found to be Prx2 for which three identified peptides matches were found (Figure 1B).

Prx2 is an antioxidative enzyme with peroxidase activity. Importantly, it also contains a conserved motif optimal for Cdks. Because of these reasons and the potential importance of ROS in PD, we chose this target for our study. To confirm the interaction between p35 and Prx2, an in vitro binding assay was carried out utilizing bacterially expressed proteins. GST-Prx2 or GST alone was incubated with His-tagged p35 and subjected to SDS-PAGE and western blot analyses using p35 antibody. A specific interaction was observed only with GST-Prx2 (Figure 1C). Similarly, a reverse binding experiment was performed where GST control or GST-Cdk5 was incubated with His-Prx2 alone or with both His-p35 and His-Prx2. Specific interaction was only observed with GST-Cdk5, His-p35, and His-Prx2 coincubation (Figure 1C). Finally, we also examined whether we could detect interaction through a means independent of bacterially expressed proteins by utilizing the yeast two-hybrid interaction assay. Consistent with our previous results, we could also detect interaction between p10 and Prx2 (Figure S1 available with this article online). These results indicate that Prx2 specifically interacted with the Cdk5/p35 complex through its association with p35.

To test whether endogenous Prx2 may exist in a complex with Cdk5/p35 in vivo, immunoprecipitation was performed using control IgG, p35 (C-19), and Cdk5 (C-8) antibodies on brain extracts (Figure 1D). The complexes were then analyzed by SDS-PAGE and Western blot analyses using a Prx2 antibody. Both immunoprecipitates using either Cdk5 or p35 antibody showed an associated

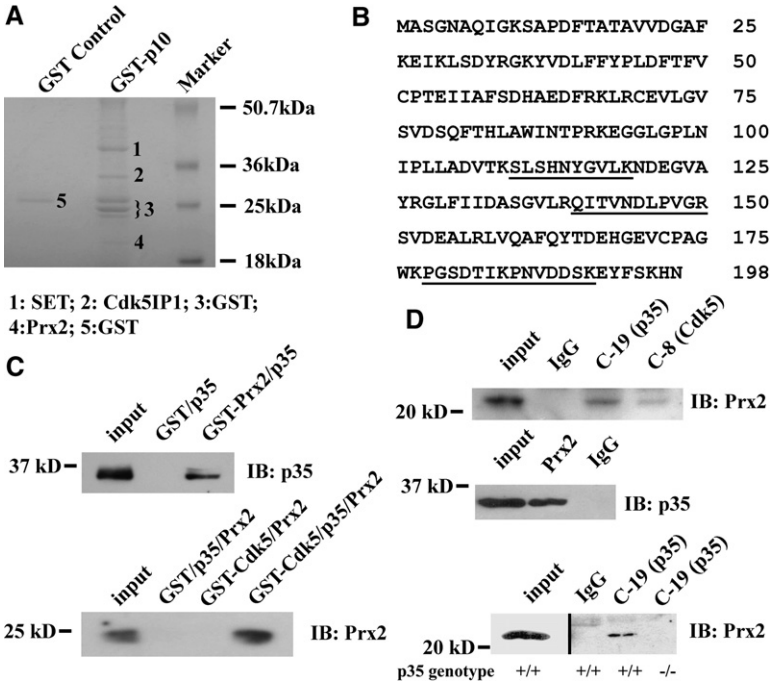

Figure 1. Identification of Prx2 as Cdk5/p35 Interactor

(A) Isolation of Cdk5-interacting proteins by affinity purification. Fifty micrograms of bacterially expressed GST and GST-p10 as bait was immobilized on GSH beads and coincubated with 1 mg of mouse brain homogenate at 4°C for 3 hr. The eluted proteins were precipitated by TCA after elution by 1 M NaCl. The precipitates were separated by 10% of SDS-PAGE then visualized by colloidal Coomassie Blue staining.

(B) Identification of Prx2 by a tandem mass spectrometry. The specific bands from GST-p10 were excised from the stained SDS-PAGE for mass spectrometric sequencing. Three underlined peptides were sequenced by the mass spectrometry and matched to mouse Prx2.

(C) Confirmation of the interaction between Prx2 and Cdk5/p35. Two micrograms of bacterially expressed GST and GST-Prx2 was incubated with 10 μg of His-p35 at 4°C for 2 hr. The GST-tagged proteins were retrieved with GSH beads. The proteins were subjected to SDS-PAGE and detected using C-19 for p35 by western blots (top panel). Similar to the above description, GST was incubated with His-p35 and His-Prx2 or GST-Cdk5 was incubated with His-Prx2 alone or with both His-p35 and His-Prx2 at 4°C for 2 hr. The bound proteins were separated by SDS-PAGE and detected by western blot using anti-Prx2 antibody after retrieval of GST-fused proteins by GSH-Sepharose (bottom panel).

(D) Association of Prx2 with Cdk5/p35 in vivo. (Top panel) Control IgG, C-19 for p35, and C-8 for Cdk5 were incubated with 500 μg of mouse brain lysate. Antibodies were isolated by IP beads and the coupled proteins were subjected to SDS-PAGE followed by anti-Prx2 western blot. (Middle panel) Control IgG or anti-Prx2 (Abcam) as indicated was incubated with mouse brain lysate. Following immunoprecipitation, samples were subjected to western blot analyses using anti-p35 antibody. (Bottom panel) Control IgG or anti-p35 were incubated with WT (+/+) or p35-deficient (−/−) mouse brain lysate as indicated. Following immunoprecipitation, samples were subjected to western blot analyses using anti-Prx2 antibody. Note that all lanes are from the same gel. However, because of the high intensity of the input-positive control signal, exposure time was reduced for this lane.

Prx2 signal by western blot while the control IgG did not. The reverse interaction assay where immunoprecipitation with Prx2 preceded western blot analyses for p35 was also performed using brain extracts. Consistent with the previous pulldown, a specific interaction between Prx2 and p35 was also observed (Figure 1D). Finally, to further confirm the specificity of the interaction assay, we also performed a p35 immunoprecipitation using p35 wild-type (WT) or knockout brain extracts followed by western blot analyses utilizing Prx2 antibody. A positive interaction was only observed with WT brain extract and not with p35-deficient brains. Taken together, this indicates that endogenous Prx2 associates with Cdk5/p35 in vivo.

Prx2 Is a Substrate of Cdk5 and Its Peroxidase Activity Is Regulated through Phosphorylation by Cdk5

The consensus sequence of Cdk5 phosphorylation is Pro (P)-directed Ser (S) or Thr (T) surrounded in the +3 position by basic amino acids, Arg (R), Lys (K), or His (H) (Songyang et al., 1996). There is a potential motif in Prx2 containing Pro-directed Thr, T^{89}PRK, optimal for Cdk5 phosphorylation. To investigate whether Prx2 is a substrate of Cdk5, Prx2 and Prx2T89A, a Prx2 mutant in which nonphosphorylatable Ala (A) replaced Thr (T), were subjected to in vitro kinase assay with Cdk5 alone, Cdk5/p35, or Cdk5/p25 active complexes. Incubation of bacterially expressed Prx2 or Prx2T89A with Cdk5 alone did not result in any radiolabel signal, indicating that the activating binding partner of Cdk5 was required for phosphorylation. The recombinant Prx2 was phosphorylated when either p35 or p25 was present along with Cdk5. In contrast, the recombinant Prx2T89A showed almost no detectable phosphorylation (Figure 2A). This indicates that both Cdk5/p35 and Cdk5/p25 can phosphorylate Prx2 and that almost all the phosphorylation occurs on the T89 residue.

To assess the effects of Cdk5-mediated Prx2 phosphorylation on peroxidase activity, WT Prx2 was purified from bacteria. It was then incubated with GST-Cdk5 and GST-p25 purified from bacteria. Afterwards, phospho-Prx2 was separated from nonphosphorylated Prx2 by Q-column. Equal amounts of phosphorylated and nonphosphorylated Prx2 were then assessed for peroxidase activity by monitoring the H_2O_2-dependent oxidation of NADPH in the presence of thioredoxin (Trx) and Trx reductase. The peroxidase activity of phospho-Prx2 was 37% of that of nonphosphorylated Prx2 (Figure 2B). To confirm isolation and phosphorylation of Prx2 as just described,

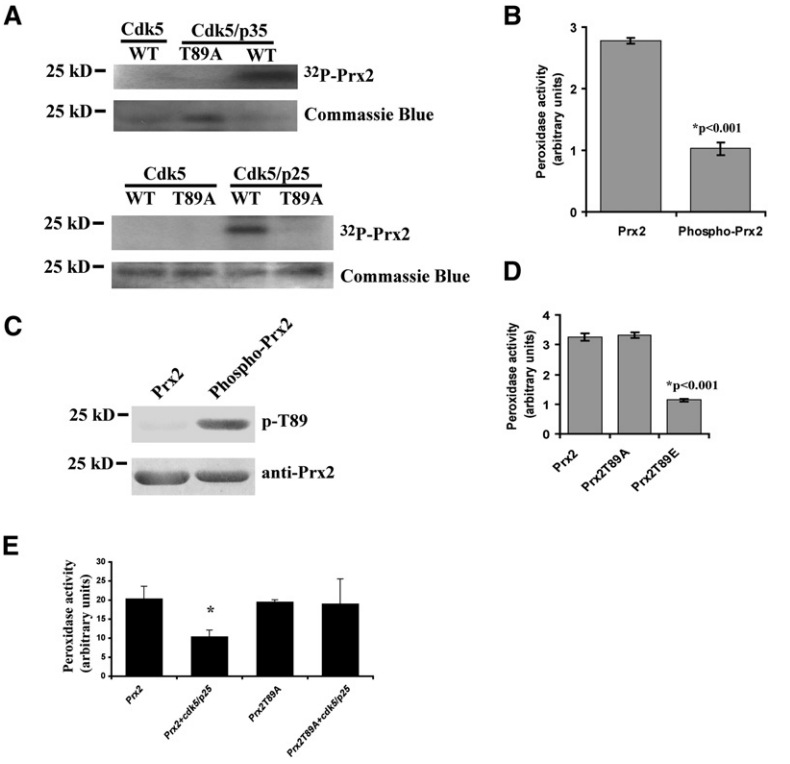

Figure 2. Prx2 Is an In Vitro Substrate of Cdk5, and Peroxidase Activity of Prx2 Is Modulated through Phosphorylation

(A) Prx2 is a substrate of Cdk5/p35 and Cdk5/p25. One microgram of purified His-Prx2 or His-PrxT89A was incubated with 50 ng of purified GST-Cdk5, GST-Cdk5/GST-p35, or GST-Cdk5/GST-p25 and 1 μCi of [γ-^{32}P]ATP at 30°C for 30 min. The proteins were separated by SDS-PAGE for autoradiography.

(B) Peroxidase activity of Prx2 is reduced by phosphorylation. Two hundred micrograms of His-Prx2 was incubated with 10 μg of GST-Cdk5/GST-p25 and 10 μM ATP at 30°C for 6 hr. The reaction mixture was separated by Q-column to isolate phospho-Prx2 from Prx2 after clearing GST-tagged Cdk5 and p25 by GSH-Sepharose. 0.5 μg of Prx2 or phospho-Prx2 was used for the peroxidase assay at 30°C for 10 min. The consumption of NADPH was measured at 340 nm wavelength by spectrophotometer. The data are the mean ± SEM (n = 3).

(C) Determination of purity of phospho-Prx2 and confirmation of specificity of p-T89 for phospho-Prx2. One microgram of Prx2 or purified phospho-Prx2 as isolated in (B) was probed by western blot analyses using the p-T89 antibody or a pan-Prx2 antibody.

(D) Peroxidase activity of Prx2T89E is lower than Prx2. 0.5 μg of purified His-Prx2, His-Prx2T89A, or His-PrxT89E was assayed for peroxidase activity as described above in (B). The data is the mean ± SEM (n = 4).

(E) Peroxidase activity of Prx2T89A is not affected by Cdk5 phosphorylation. Ten micrograms of purified His-Prx2 or His-Prx2T89A was incubated with or without GST-Cdk5/GST-p25 (3 μg each) overnight at 30°C in the presence of 2 mM DTT. Following dialyses, peroxidase activity was measured as described in (B). The data are the mean ± SEM (n=3).

we generated a phospho-antibody specific for phosphorylated T89 of Prx2. The phospho-T89 (p-T89) antibody recognized phosphorylated Prx2 but not the nonphosphorylated form isolated by Q-column upon western blot analyses (Figure 2C). A pan-Prx2 antibody was also isolated and shows that total Prx2 levels were approximately equal between nonphosphorylated and phosphorylated forms (Figure 2C).

The above results indicate that Cdk5 phosphorylation of Prx2 results in downregulation of peroxidase activity. To confirm this and ascertain that this is due to phosphorylation at T89, we isolated bacterially expressed Prx2, the Prx2T89A mutant, and a Prx2T89E mutant designed to mimic phosphorylation. Equal amounts of proteins were assayed for peroxidase activity. Importantly, the mutant Prx2T89E resulted in a 66% reduction in peroxidase activity compared to Prx2. The mutant Prx2T89A on the other hand was not significantly different from WT (Figure 2D). Importantly, we would predict that if Cdk5-mediated phosphorylation of Prx2 at T89 leads to downregulation of peroxidase activity, the Prx2T89A mutant should not be responsive to Cdk5 phosphorylation. Consistent with this notion, phosphorylation of recombinant WT Prx2 but not the Prx2T89A mutant by Cdk5 in vitro led to reduced peroxidase activity (Figure 2E). Finally, we also observed that phosphatase treatment of WT Prx2 previously phosphorylated by Cdk5 reversed the decrease in peroxidase (see Figure S2). Taken together, our data, at least in vitro, indicate that Cdk5 phosphorylates Prx2 at T89, which results in reduced Prx2 activity.

Prx2 Plays a Protective Role in Cortical Neurons Insulted by Neurotoxin MPP$^+$

We next determined whether Prx2 and its phosphorylation may play a role in neuronal death induced via mitochondrial stress by evaluating the effects of MPP$^+$, the active metabolite of MPTP, on death of cultured cortical neurons. It is important to note that these cultures are completely neuronal as evaluated by MAP2 staining (see Figure S3). We first evaluated the effect of MPP$^+$ on T89 phosphorylation utilizing the phospho-specific antibody p-T89 for phosphorylated Prx2 at T89. Analysis of an MPP$^+$ time course by western blot indicated a maximal increase in phospho-Prx2 signal at 24 hr (Figure 3A). This coincided with the peak in Cdk5 kinase activity measured under the same conditions (Figure 3B). In the latter assay, immunoprecipitated Cdk5 from neurons treated by MPP$^+$ at different time points was subjected to a kinase assay using histone H1 as a substrate. The increase in Prx2 phosphorylation at T89 was also observed in cortical neurons analyzed by immunofluorescence using the same phospho-T89 antibody after MPP$^+$ treatment (Figures 3C, 3D,

and 3E). There was a notable increase in phospho-labeling upon MPP^+ stress in the soma and neurites. This increase of fluorescence was quantified over a number of neurons by image analyses, showing a 90% increase in neurites and 62% increase in the soma. Blocking peptide treatment was used as a control for specificity and shows the required loss of fluorescent signal. Importantly, levels of Prx2 did not dramatically change upon MPP^+ treatment. This was observed both with western blot (Figure 3A) and upon immunofluorescent analyses (Figure 3C). Similar observations of increased phosphorylation of Prx2 were also observed in midbrain cultures containing dopamine neurons exposed to MPP^+ (see Figure S4). In addition, we also observed Prx2 phosphorylation when midbrain neurons were treated with another mitochondrial toxin, rotenone (Greenamyre et al., 2003) (see Figure S5).

We next determined whether a reduction in Prx2 activity accompanied the increase in T89 phosphorylation by analyzing a time course following MPP^+ treatment of cortical neurons. Importantly, Prx2 activity decreased at 24 hr, opposite to that of T89 Prx2 phosphorylation and Cdk5 activation (Figure 3F). These results suggest that Prx2 peroxidase activity is regulated by T89 phosphorylation in cultured neurons following mitochondrial insult.

To evaluate whether T89 phosphorylation of Prx2 plays a role in neuronal death induced by MPP^+, cortical neurons were infected with virus expressing Prx2, Prx2T89A, and Prx2T89E. The viability of the infected neurons was assessed by evaluating nuclear integrity after exposing cultures to MPP^+ for 48 hr. Expression of Prx2 and Prx2T89A significantly protected neurons from death in comparison to that of GFP and Prx2T89E (Figure 3G). Conversely, we also evaluated whether downregulation of Prx2 might sensitize neuronal cultures to MPP^+ treatment. We designed three different siRNA sequences to Prx2. The siRNA sequences 1 and 2 showed the most significant reduction in Prx2 levels (Figure 3H). These siRNA sequences sensitized the neuronal cultures to the toxic effects of MPP^+ treatment (Figure 3H). Finally, we also examined for ROS under these conditions by 2′, 7′-Dicholororescein diacetate (DCF) staining. As shown in Figure 3I, both siRNA sequences 1 and 2 significantly increased the number of DCF-positive neurons. Taken together, our results suggest a model by which a decrease of Prx2 peroxidase activity mediated through T89 phosphorylation after MPP^+ insult enhances oxidative stress, resulting in neuronal death.

T89 Phosphorylation of Prx2 Is Mediated by Cdk5 in Neurons after MPP^+ Treatment

To investigate whether T89 of Prx2 is phosphorylated in neurons by Cdk5, lysates from WT and $p35^{-/-}$ neurons treated with MPP^+ were analyzed by western blot. Absence of p35 led to a significant decrease of phosphorylation of Prx2 at T89 (Figure 4A). It is important to note, however, that this reduction was not absolute, suggesting that, at least in the present in vitro paradigm, other activators of Cdk5 such as p39 might also be present. It might also be due to the actions of other kinases. Prx2 levels were not observed to vary between WT and $p35^{-/-}$ neurons with or without MPP^+ treatment. To further confirm this reduction in phospho-Prx2 signal with p35 deficiency, we carried out immunofluorescent analyses. In $p35^{-/-}$ neurons, the increase in phospho-T89 signal observed upon MPP^+ treatment in WT neurons was significantly reduced (Figure 4B). Consistent with this observation, similar inhibition of Prx2 phosphorylation was observed with treatment of the Cdk inhibitor Roscovitine, but not with the GSK3 inhibitor lithium (see Figure S6). These data indicate that the phosphorylation of Prx2 at T89 in neurons following MPP^+ insult is significantly dependent upon Cdk5 activity. Next, we examined peroxidase activity of Prx2 in WT and $p35^{-/-}$ neurons following MPP^+ treatment. The data clearly showed that Prx2 peroxidase activity was decreased following MPP^+ treatment in WT neurons. However, this reduction did not occur in $p35^{-/-}$ neurons (Figure 4C). This indicates that peroxidase activity of Prx2 is regulated via phosphorylation by Cdk5. These data also suggested that $p35^{-/-}$ neurons should show reduced oxidative stress and resistance to MPP^+-induced neuronal death. To test this, ROS levels were measured utilizing DCF. ROS was significantly increased in WT neurons upon MPP^+ exposure as measured by both average intensity (54% increase) (Figures 4E and 4F) and number of DCF-positive neurons (34% increase) (data not shown). In contrast, $p35^{-/-}$ neurons showed reduced ROS levels upon MPP^+ treatment (35% decrease average density, Figure 4F; 25% decrease total number of cells, data not shown) when compared to WT littermate controls. $p35^{-/-}$ neurons were also substantially protected from MPP^+-induced death when compared to WT littermate control neurons (Figure 4D). Finally, based upon our previous observations that calpain-mediated activation of Cdk5 is important for neuronal death (Smith et al., 2006), we would predict that calpain inhibition should also block Prx2 phosphorylation. Consistent with this, cotreatment of MPP^+-exposed neuronal cultures with the calpain inhibitor PD150606 (Sedarous et al., 2003; Wang et al., 1996) led to decreased Prx2 signal as measured by immunofluorescence analyses (Figures 4G and S7). Taken together, our data indicate that Cdk5 kinase activity has a critical role in the reduction of Prx2 peroxidase activity through phosphorylation of Prx2 at T89 following MPP^+ insult and that this is a contributing factor to ROS increase and the ensuing neuronal death.

Prx2 Prevents the Loss of DAergic Neurons in the SNc in MPTP Mouse Model of PD

The phosphorylation of Prx2 at T89 by Cdk5 has a functional role in neuronal death in the MPP^+-induced cell death model. These data led us to further investigate the effects of Prx2 on neuronal death in an in vivo mouse model of PD. We employed the MPTP mouse model of PD to assess roles of Prx2 in the loss of DAergic neurons. We first determined the effects of expression of WT Prx2 and its mutants, Prx2T89A and Prx2T89E, on survival of DAergic neurons following MPTP administration. These

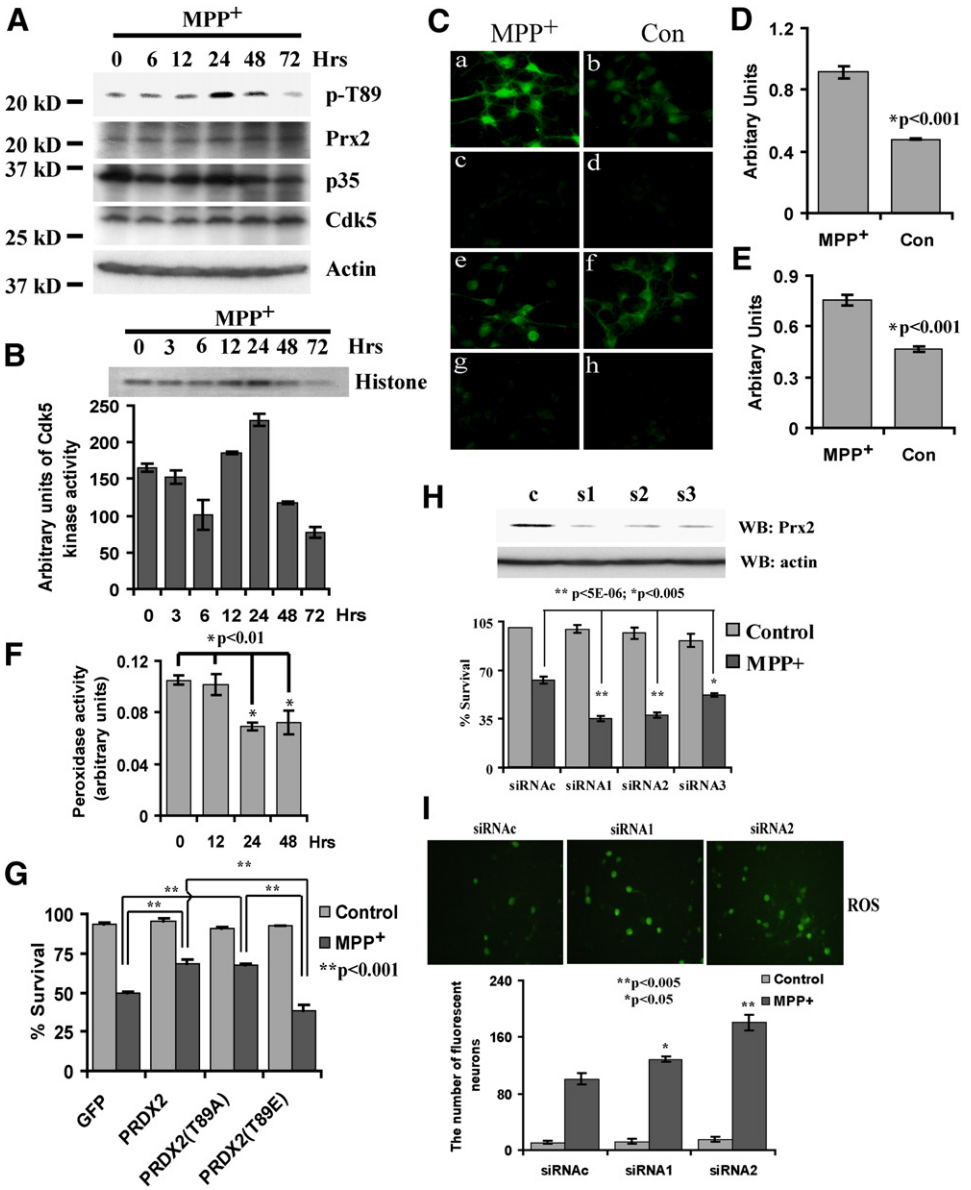

Figure 3. Reduction of Prx2 Peroxidase Activity and Associated Phosphorylation at T89 in Neurons after MPP$^+$ Treatment

(A) Increase of phospho-Prx2 after MPP$^+$ insult. Cultured cortical neurons were treated with 20 μM MPP$^+$. The treated neurons were harvested at different time points, 6, 12, 24, 48, or 72 hr after MPP$^+$ treatment. Forty micrograms of cell lysate was analyzed by western blot analyses utilizing p-T89, anti-Prx2, C-19 for p35, C-8 for Cdk5, or anti-β-actin antibodies. Similar results were observed in three independent experiments.

(B) Increase of Cdk5 kinase activity in neurons after MPP$^+$ treatment. Neurons were treated as above described. Cdk5 was isolated from 50 μg of cell lysate by immunoprecipitation using the C-8 antibody and was incubated with 0.5 μCi of [γ-^{32}P]ATP at 30°C for 30 min using histone H1 as a substrate. The proteins were separated by SDS-PAGE for autoradiography. The density of autoradiographic bands was normalized from three experiments and is presented as mean ± SEM.

(C) Increase of phospho-Prx2 in neurons by immunofluorescent staining. After 24 hr treatment by MPP$^+$, neurons were fixed by 4% paraformaldehyde. (a–d) Neurons were incubated with p-T89 antibody and coincubated with the appropriate phosphorylated peptide sequence used to produce the antibody (c and d) or the control nonphosphorylated peptide sequence (a and b). (e–h) Neurons were fixed as above and stained with our pan-Prx2 antibody without (e and f) or with (g and h) a quenching peptide used to produce the antibody as control. The images were captured by fluorescent microscopy.

(D and E) Distribution of phospho-Prx2 in neurons treated by MPP$^+$. The fluorescent signal in soma (D) or dendrites (E) was measured from 200 neurons through image analysis. The data are the mean ± SEM.

(F) Downregulation of Prx2 peroxidase activity after MPP$^+$ treatment. Prx2 was isolated from cultured neurons treated with MPP$^+$ for the indicated times using a monoclonal anti-Prx2 antibody obtained from Abcam. Peroxidase activity was measured as described above. The data are the mean ± SEM (n = 3).

constructs were targeted unilaterally to the SNc DAergic neurons using an adenoviral-mediated gene delivery approach as we have previously performed (Crocker et al., 2001, 2003; Kalia et al., 2004; Kim et al., 2005; Smith et al., 2003). Protein expression of adenoviral Prx2 and its mutant was verified by western blot analysis (Figure 5B). We found that expression of Prx2 and Prx2T89A resulted in significant DAergic neuroprotection following MPTP treatment when compared with the contralateral untreated side or with GFP-injected control animals as analyzed by counting the number of tyrosine hydroxylase (TH) positive neurons (Figures 5A and 5B). In contrast, expression of Prx2T89E showed similar effects to GFP controls (Figures 5A and 5B). As an independent assessment of survival, we also examined for the number of neurons in the SNc region by cresyl violet staining. This also ensures that loss of neurons by MPTP treatment is not simply due to loss of TH expression. Our cresyl violet analysis was similar to that of TH assessment (Figure 5C). These data indicate that Prx2 can modulate DAergic neuron survival in the SNc following MPTP administration and suggest that the T89 regulatory site may be important in this DAergic cell death process.

Cdk5-Mediated Phosphorylation of Prx2 at T89 Plays a Pivotal Role in DAergic Neuron Damage by Regulation of Prx2 Peroxidase Activity in an MPTP Mouse Model of PD

The above in vivo evidence only demonstrates that Prx2 could potentially be important in the MPTP model. To further support this, we examined whether endogenous Prx2 may be modulated at T89 following MPTP treatment. Accordingly, the SNc extracts obtained from mice treated with MPTP for various times were subject to western blot analyses. As shown in Figure 6A, Prx2 phosphorylation increased following MPTP treatment, reaching the highest level of phosphorylation at 3 days following injected MPTP. To determine whether the phosphorylation of Prx2 at T89 is mediated by Cdk5, the SNc lysates from $p35^{-/-}$ mice or WT littermate controls were treated with MPTP and analyzed 3 days post-treatment. The level of phosphorylated Prx2 in $p35^{-/-}$ mice was significantly less than that in WT mice after MPTP treatment (Figure 6B). To further confirm the increase in the level of phosphorylated Prx2 in DAergic neurons in SNc of MPTP-treated mice, we assessed Cdk5-phosphorylated Prx2 by immunofluorescence analyses. Phosphorylated Prx2 was clearly observed in DAergic neurons in the SNc of WT mice treated with MPTP but not substantially observed in that of $p35^{-/-}$ mice (Figure 6C). We quantified the average fluorescent signal from TH-positive neurons by image analyses. As shown in Figure 6D, the fluorescent signal of phospho-Prx2 in WT SNc increased approximately 30%–50% in comparison to untreated WT controls or treated and untreated $p35^{-/-}$ mice.

To examine whether the peroxidase activity of Prx2 is affected in the MPTP mouse model of PD, Prx2 was isolated from $p35^{-/-}$ SNc or WT littermate controls with and without MPTP treatment and assayed for activity. Prx2 isolated from WT mice treated by MPTP showed a significant decrease in peroxidase activity in comparison to untreated WT controls. In contrast, $p35^{-/-}$ animals did not show this reduction following MPTP treatment (Figure 6E). Finally, we have previously shown that calpains are central for Cdk5 activation (Smith et al., 2006). Consistent with this, adenoviral-mediated expression of the calpain inhibitor calpastatin blocks increase in phospho-Prx2 following MPTP treatment in vivo (Figure S8). Taken together, these data indicate that calpain-mediated Cdk5 activation mediates phosphorylation and reduction of Prx2 activity in an in vivo model of PD and that this activity plays an important role in the death of DAergic neurons.

Relevance to Human PD

While the above evidence strongly implicates the importance of Prx2 in the MPTP model of PD, we directly examined its potential relevance to human PD. Accordingly, we first examined the phospho-Prx2 signal in human PD postmortem samples and controls. The equivalent T89 site is also present in human Prx2. As shown in Figure 7, nigral DAergic neurons from human midbrain PD and control samples were clearly detected by the presence of neuromelanin, granular brown pigmented regions detectable even without staining (see arrowheads in Figures 7A and 7B). When stained using phospho-Prx2 antibody and DAB visualization, little or no signal was detected in the perikarya of dopamine neurons from control midbrain samples. However, significant staining (black color) was observed in soma of dopamine neurons from PD patients (see arrow, Figure 7A). Staining in the region of neurons that resemble dopamine neurons in size and location but that did not contain neuromelanin was also observed in

(G) Prx2 protects neuron form death after MPP^+ treatment. Cortical neurons were infected with virus expressing GFP alone or along with Prx2, Prx2T89A, or Prx2T89E and cultured for 3 days. The cells were exposed to MPP^+ for 48 hr and neuronal survival was evaluated by assessing nuclear integrity of GFP-positive neurons. The data are the mean ± SEM (n = 3). Similar results were obtained when equivalent constructs were transfected (data not shown).

(H) Downregulation of Prx2 by siRNA oligonucleotide treatment sensitizes cortical neurons to MPP^+ treatment. Three independent siRNA sequences (s1/siRNA1, s2/siRNA2, s3/siRNA3) and a control sequence (c/siRNAc) were evaluated for their ability to downregulate endogenous Prx2 levels in cortical neurons as described in Experimental Procedures (top panel). (Bottom graph) Transfected cultures were then assayed for survival following 48 hr MPP^+ treatment by MTT assay. The data are the mean ± SEM (n = 3).

(I) Downregulation of Prx2 by siRNA oligonucleotide treatment increases ROS levels following MPP^+ treatment. (Top panel) Representative DCF fluorescence in control (siRNAc) or siRNA1 or siRNA2 oligonucleotide-treated cultures after treatment with 20 μM MPP^+ for 24 hr under a fluorescent microscope. (Bottom graph) Quantification of the number of DCF fluorescence-positive cells in cells treated as above. Random fields were analyzed for the number of DCF-positive neurons. The data are the mean ± SEM (n = 4).

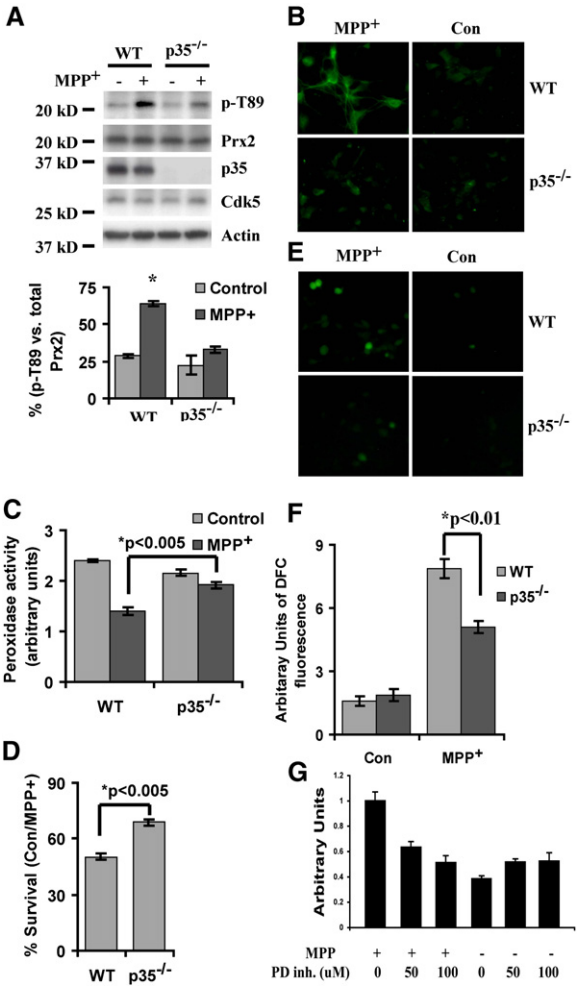

Figure 4. Prx2 Is a Substrate of Cdk5 in Neurons Treated with MPP+

(A and B) Cdk5 phosphorylates Prx2 in neurons treated by MPP+. (A) Neurons from WT or p35$^{-/-}$ embryos were treated with MPP+ insult for 24 hr. The cell lysates were subjected to SDS-PAGE and p-T89 western blot analyses. The membranes were then stripped and reprobed with anti-Prx2, C-19 for p35, C-8 for Cdk5, or anti-β-actin. The bottom panel shows densitometric values of phospho-Prx2 relative to Prx (p-T89/total Prx2*100). Each value is the mean ± SEM (n = 3). (B) Likewise, cultures as indicated were subjected to immunofluorescent staining utilizing the p-T89 antibody. Similar results were obtained in three independent experiments.

(C) Phosphorylation of Prx2 at T89 by Cdk5 reduces peroxidase activity. Cortical cultures from WT or p35$^{-/-}$ embryos were treated with MPP+ for 24 hr as described above. Peroxidase activity assay was carried out also as described above. The data are the mean ± SEM (n = 3).

(D) p35$^{-/-}$ neurons are resistant to MPP+-induced death. Neurons from WT or p35$^{-/-}$ embryos were exposed to MPP+ for 48 hr. The viability of neurons was measured by MTT assay. The survival percentage was obtained by comparing value from the MPP+-treated neurons to that of the nontreated neurons in either p35$^{-/-}$ or WT neuronal cultures. The data are presented as mean ± SEM (n = 3).

(E and F) The role of Cdk5/p35 in MPP+-induced ROS. (E) Representative DCF fluorescence in WT and p35$^{-/-}$ neurons after treatment with 20 μM MPP+ for 24 hr under a fluorescent microscope. (F) Quantification of DCF fluorescence signal in WT and p35$^{-/-}$ neurons either treated or untreated with MPP+. Random fields were analyzed for average fluorescence intensity. The data are the mean ± SEM (n = 4).

(G) Calpain inhibitors block increase in MPP+-induced phospho-Prx2 signal. Cortical neuronal cultures were untreated or treated with 20 μM MPP+ and/or the calpain inhibitor PD150606, as indicated. Cultures were fixed and stained for phospho-Prx2 and Hoechst. For representative pictures, please see Figure S7. The fluorescent signal in soma was measured by image analyses from 45 neurons in 3 random fields. The data are presented as mean ± SEM.

PD samples. Staining of neuritic processes was observed in both control and PD samples. The number of phospho-Prx2-positive dopamine neurons was then quantified from five PD and six control samples. As shown in Figure 7C, a significant increase in phospho-Prx2-positive neurons was observed in PD patient samples when compared to controls.

Finally, we examined whether familial PD genes may impact Prx2 phosphorylation. The PD gene, *dj-1*, has also been linked to management of ROS (Bonifati et al., 2003; Kim et al., 2005). Interestingly, DJ-1 expression blocked Prx2 phosphorylation in neurons treated with MPP+ (Figure S6C). Modulation of another PD gene, *pink1* (Valente et al., 2004), however, did not affect Prx2 phosphorylation, suggesting some specificity in the way PD genes impact Prx2 phosphorylation (Figure S6D). Taken together, our human patient data as well as that with DJ-1 further support the importance of Prx2 in PD.

DISCUSSION

ROS are generated as a result of normal metabolism (Adam-Vizi, 2005). However, generation of excessive oxidative load beyond a cell's homeostatic capacity can be deleterious. Mitochondrial dysfunction and excess ROS have been strongly implicated in the pathogenesis of PD (Jenner, 1998; Przedborski, 2005). However, how these events are initiated, are regulated, and interact to promote neuronal death is not completely clear. Recently, we demonstrated that calpain-mediated Cdk5 activation plays an essential role in DAergic loss in the MPTP model of PD (Crocker et al., 2003; Smith et al., 2003, 2006). These findings were important since they provided a plausible link between the actions of a mitochondrial damaging agent (MPTP) and activation of a pathogenic calcium-dependent process (calpain activation) consistent with known deregulation of calcium homeostasis in PD. However, the manner by which Cdk5 regulates downstream pathogenic events was not completely known. Presently, we identified a novel Cdk5 target, Prx2, an antioxidant enzyme with peroxidase activity (Rhee et al., 2005). We provide evidence that Prx2 is a physiological substrate of Cdk5. Cdk5 activation downregulates Prx2 peroxidase activity in PD models of death both in culture and in animals. Modulation of Prx2 activity also regulates neuronal loss. These data provide a mechanistic link of how the mitochondrial damaging agent MPTP leads to nigral loss by Cdk5 activation, phosphorylation/inactivation of an

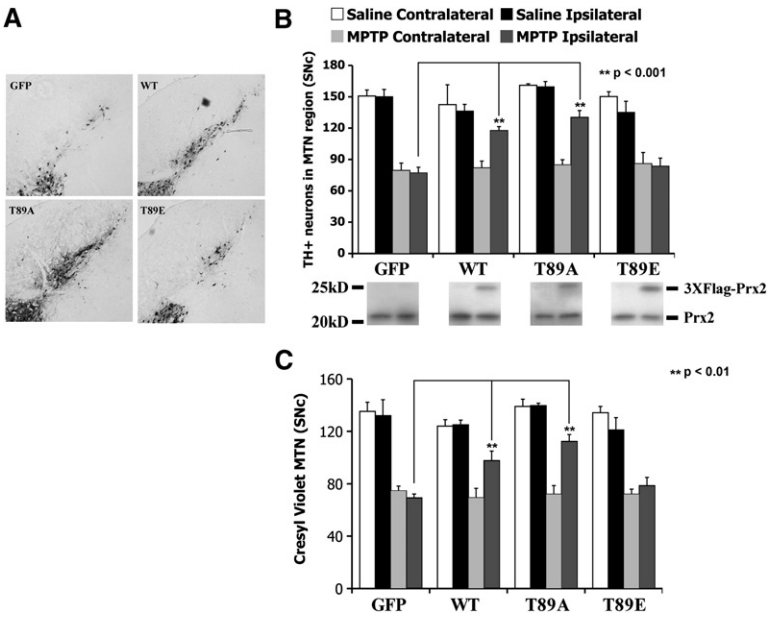

Figure 5. Prx2 Protects DAergic Loss following MPTP Administration

(A) The adenoviruses (2 μl, 1 × 10^7 particles per μl) expressing Prx2, Prx2T89A, and Prx2T89E were injected directly into the striatum of animals 7 days before initiation of MPTP treatment. A GFP-expressing virus was used as a control. Brains were sectioned into 14 μm slices for TH DAB staining. Representative pictures of the ipsilateral side of animals injected with the indicated virus and treated with MPTP were shown.

(B) Quantification of the number of DAergic neurons from ipsilateral or controlateral for the indicated treatment groups are shown. The data are presented as mean ± SEM (n = 4–5/group). Expression of Prx2, Prx2T89A, and Prx2T89E in the SNc extracts was confirmed by western blot analyses using a pan-antibody for Prx2.

(C) Quantitation of neurons in the SNc region by cresyl violet staining. The data are presented as mean ± SEM (n = 4/group).

important antioxidant enzyme, and consequent increase in oxidative load (see Figure 8). The observation of increased Prx2 phosphorylation in human PD tissue as well as modulation by DJ-1 also indicates the potential importance of this pathway in human PD.

Prx2 Interacts with Cdk5/p35 Complexes and Is a Substrate of Cdk5

Our results demonstrate that Cdk5/p35 interacts with Prx2. Prx2 is a member of the Prx family that contains at least six members. The identification of the Prx2 form as an interacting partner is particularly relevant since Prx2 is localized to neurons, including the DAergic neurons of the SNc (Jin et al., 2005; Sarafian et al., 1998). This is, in turn, consistent with known DAergic functions of Cdk5, particularly in models of PD as reported previously (Smith et al., 2003, 2006). In contrast, Prx1, also localized to the cytoplasm, is distributed in oligodendrocytes and microglia (Jin et al., 2005). These results, particularly in vivo, point to a neuron-specific Cdk5-Prx pathway of ROS management rather than a non-cell-autonomous mode of action regulated by other brain cell types such as glia. It is important to point out that this does not exclude the potential importance of other Prx members in neuronal loss. For example, Prx3, a mitochondrially localized enzyme (Watabe et al., 1994), also has potential Cdk5 sites. It will be interesting to determine whether this member might also play a Cdk5-dependent role in mitochondrial stress-induced death.

Our initial results indicated that the N-terminal portion of p35 was sufficient to bind to Prx2. However, it is important to note that both p35 and p25 can efficiently phosphorylate Prx2, at least in vitro. This suggests that stable binding to a Cdk5/p35 complex per se mediated by the p10 fragment is not required for efficient phosphorylation. We speculate that the p10 portion may be an important regulatory domain that regulates how efficiently p35 or Cdk5/p25 complexes may phosphorylate Prx2. Careful analyses will have to be performed to further study this interesting observation. Nonetheless, our results indicate not only that both Cdk5 complexes phosphorylate Prx2 on T89, but also that this modification significantly downregulates its activity.

Under basal conditions, p35 is abundantly localized to the inner cellular membrane, through a myristoylation anchor (Patrick et al., 1999). Appropriate activation of this form of Cdk5/p35 is the presumptive "normal" activity of this complex. However, p35 can be converted to a pathogenic p25 form by calpain-mediated cleavage (Lee et al., 2000; Smith et al., 2003, 2006). This results in a more stable active Cdk5 activator as well as the potential to be mislocalized to the nucleus (Gong et al., 2003; O'Hare et al., 2005). One suggested nuclear target of the Cdk5/p25 complex is Mef2, which we and others have shown is important in models of oxidative stress in vitro (Gong et al., 2003) and following MPTP in vivo (Smith et al., 2006).

Cytoplasmic Cdk5 activity might also be pathogenic. For example, a portion of p25 could also be localized to the cytoplasm, and cytoplasmic targets such as tau have been previously proposed for Cdk5 particularly in models of Alzheimer's disease (Patrick et al., 1999). In this regard, we have identified an important cytoplasmic target of Cdk5 that could be regulated by either Cdk5/p35 or Cdk5/p25 complexes. The observation that Cdk5/p25 complexes efficiently phosphorylate Prx2 on T89, however, is consistent with a pathogenic role of this complex.

Cdk5-Mediated Prx2 Downregulation in PD and Oxidative Stress

The identification of Prx2 as a target of Cdk5 is particularly relevant since DAergic neurons are thought to be

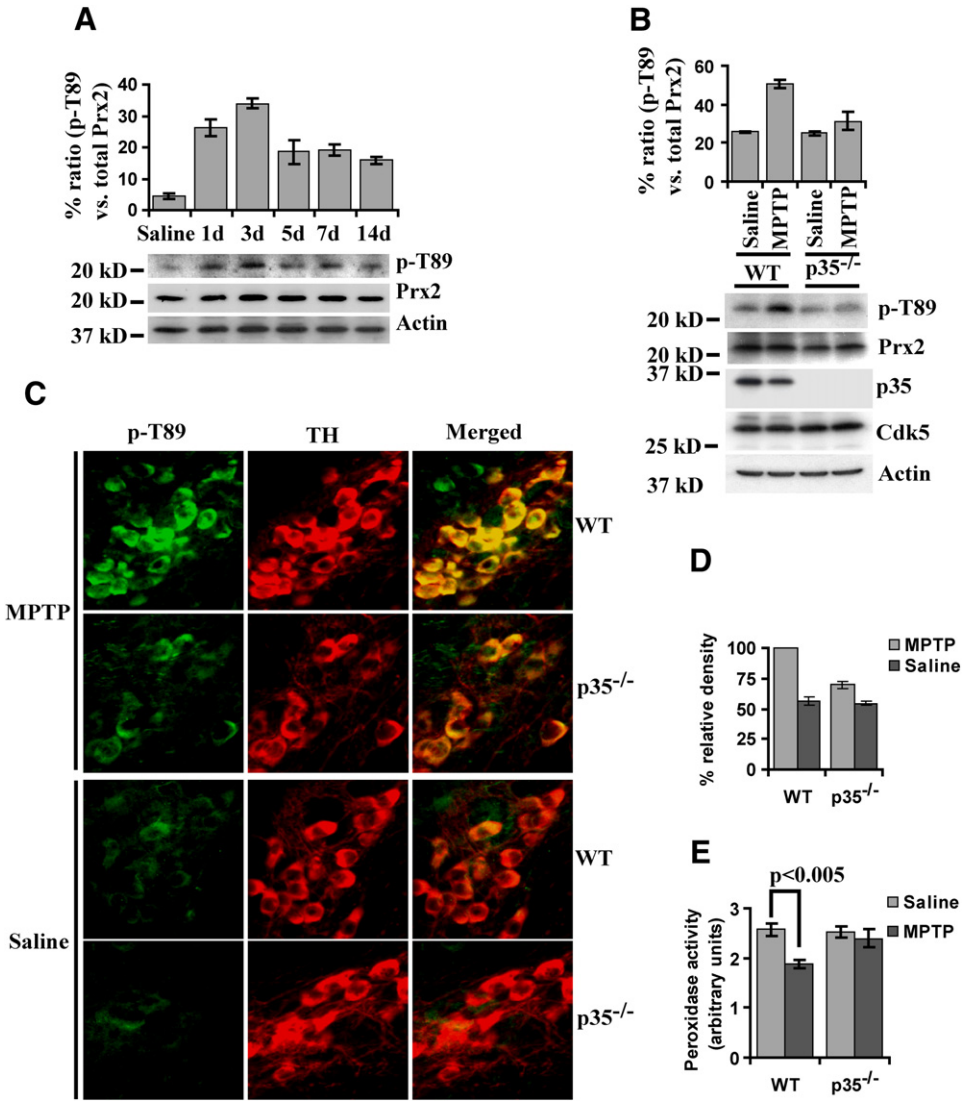

Figure 6. Peroxidase Activity of Prx2 Is Regulated in an In Vivo MPTP Mouse Model of PD

(A) Phosphorylation of Prx2 at T89 is increased after MPTP administration. The SNc extracts were obtained from animals treated with MPTP for the indicated times. (Bottom panel) The SNc lysates were subjected to SDS-PAGE and western blot probed as indicated using p-T89, anti-Prx2, and anti-β-actin antibodies. The top panel shows densitometric values of phospho-Prx2 relative to Prx (p-T89/total Prx2*100). Each value is the mean ± SEM (n = 3).

(B) Prx2 is a substrate of Cdk5 complexes in DAergic neurons from MPTP-administrated mice. (Bottom panel) The SNc lysates from WT or $p35^{-/-}$ mice 3 days following MPTP or saline administration were analyzed by western blot analyses using p-T89, anti-Prx2, anti-p35 (C-19), anti-Cdk5 (C-8), and anti-β-actin antibodies. The top panel shows densitometric values of phospho-Prx2 relative to Prx (p-T89/total Prx2*100). Each value is the mean ± SEM (n = 3).

(C) Increased phospho-Prx2 is colocalized with TH-positive neurons. The sections from WT or $p35^{-/-}$ mice were analyzed 3 days following MPTP or saline treatment. Sections were double-stained using p-T89 antibody (green) and anti-TH monoclonal (red) antibody for 24 hr at 4°C. The sections were incubated with Alex-488-conjugated antibody specific for rabbit IgG and Alex-594-conjugated antibody for mouse IgG for 3 hr at room temperature. The sections were visualized by fluorescent microscopy.

(D) The fluorescent signals from the p-T89 labeling (C) were quantified densitometrically by imaging analysis. Three sets of animals (n = 1 animal/treatment group/set) were individually stained and analyzed by densitometric analyses. 40–50 TH-positive neurons for each animal (over 3–6 slides/animal) were measured for p-T89 signal. This value was then averaged. Within each set of animals, the average value for each treatment group was normalized to the WT MPTP value. The values were then averaged for all three sets of animals for an n = 3 (mean ± SEM).

(E) Downregulation of peroxidase activity of Prx2 is mediated by Cdk5 in mice administrated by MPTP. Prx2 was isolated from 50 μg of SNc lysates from WT or $p35^{-/-}$ mice obtained 3 days following treatment with saline, or MPTP was analyzed for peroxidase activity. The data are presented as mean ± SEM (n = 3).

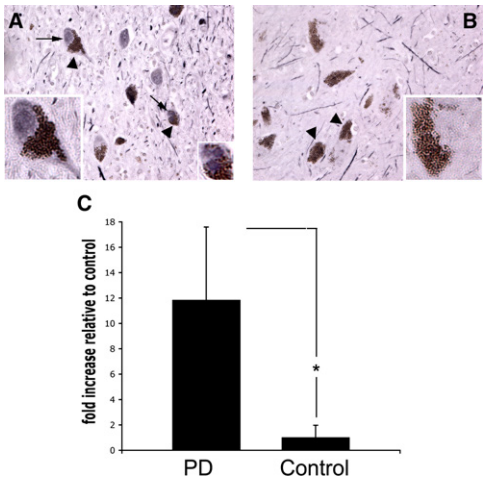

Figure 7. Prx2 Phosphorylation in Human PD

Phosphorylation of Prx2 is increased in human PD. Human substantia nigra obtained from (A) PD and (B) control individuals. Sections were immunostained using p-T89 Prx2 antibody and visualized by DAB staining. Neuromelanin pigment indicative of dopamine nigral neurons is present as punctate brown staining (arrowheads) while the phospho-Prx2 signal shows as black staining in the soma (arrows). Two examples of phospho-Prx2 positive (A) and negative (B) neurons are labeled with arrows/arrowheads. (C) Quantitation of phospho-Prx2-positive neurons in PD (n = 5) and control (n = 6) individuals are shown (mean ± SEM). *$p < 0.05$ (Student's t test).

particularly susceptible to oxidative stress (Smythies and Galzigna, 1998). There are several lines of evidence that support a link between ROS and PD. For example, ROS levels are very high in PD patients (Jenner, 1998; Przedborski, 2005). Numerous enzymes that produce ROS have been implicated as critical in in vivo models of PD (Przedborski, 2005). Damaging ROS has also been shown to occur in animals following exposure to mitochondrial poisons (Ara et al., 1998; Schapira, 2001). Importantly, the mitochondria, a major source of oxidative stress, participates in PD pathogenesis (Przedborski, 2005). Consistent with this notion, familial forms of PD have been associated with mitochondrial dysfunction. Indeed, the familial PD gene *dj-1* is thought to possess direct antioxidant functions (Bonifati et al., 2003; Canet-Aviles et al., 2004; Dawson and Dawson, 2003; Kim et al., 2005; Martinat et al., 2004; Shendelman et al., 2004).

Our evidence suggests that regulation of Prx2 is important in a toxin model of PD. For example, alteration of Prx2 levels modulates death both in vitro and in vivo following MPP^+/MPTP. It must be noted, however, that there are limitations to relating the in vivo MPTP model to PD and caution must be observed in making any direct comparisons to the human condition. For example, the relatively acute toxic nature of the MPTP model might not reflect accurately what occurs in the idiopathic PD. Accordingly, to support our MPTP data, we also report that phosphorylation of Prx2 also occurs in the nigral region of PD patients. This is consistent with the relevance of our findings to the human condition. However, standard and important caveats to interpreting any postmortem data apply here as well.

Using the MPTP model as an important first step in understanding the nigral degenerative process, we have identified how Prx2 is modulated to promote death following exposure to this mitochondrial toxin. We have shown previously that Cdk5 is hyperactivated and plays a major functional role in dopamine loss in the MPTP model (Smith et al., 2003). It is likely that Cdk5 acts to modify several downstream targets. For example, we had also previously shown that Cdk5 targets the nuclear transcription factor and survival factor Mef2 on a site known to suppress its activity (Smith et al., 2006). However, cytoplasmic targets may also be critical. We believe that Prx2 is one such important cytoplasmic factor. This is supported by our data showing that Prx2 is phosphorylated at T89 both in vitro and in vivo following MPP^+/MPTP and that this is associated with a decrease in peroxidase activity. In support of

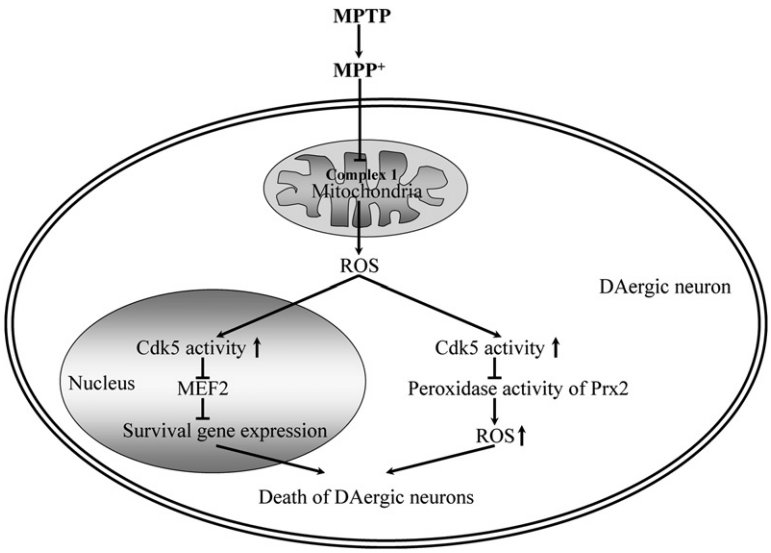

Figure 8. Model of Cdk5-Mediated DAergic Loss in MPTP Mouse Model of PD

Cdk5 kinase activity is activated by mitochondrial stress induced by MPP^+, a metabolite of MPTP. Activated Cdk5 regulates DAergic loss through phosphorylation of cytoplasmic substrate Prx2 to inhibit its antioxidative ability and phosphorylation of nuclear target Mef2 to inhibit Mef2 prosurvival function.

this, a mutant mimicking constitutively phosphorylated Prx2T89E does not protect neurons from mitochondrial insult, whereas WT or a mutant lacking the T89 phosphorylation site effectively promotes survival. Most importantly, Prx2 phosphorylation is dependent on the Cdk5 complex since p35-deficient animals, which are resistant to death induced by MPP^+ or MPTP, have reduced Prx2 phosphorylation and Prx2 peroxidase activity. It is important to highlight that, in addition to peroxidase activity, Prx2 is also thought to possess some chaperone activity at least in cell lines (Moon et al., 2005). The relevance of this in the present context is not completely known. However, we have determined that the higher molecular weight complexes of Prx2 indicative of its chaperone activity do not change following MPP^+ insult, suggesting that its chaperone activity may not be relevant in this model (D.Q. and D.S.P., unpublished results). Finally, as mentioned previously, whether other members of Prx may be important in nigral degeneration is unknown. Intriguingly, Prx1 has been shown to be phosphorylated by cell-cycle Cdk members (Yang et al., 2002). The latter has been also implicated in neuronal death (Bu et al., 2002; Busser et al., 1998; McShea et al., 1997; Nguyen et al., 2003; Osuga et al., 2000; Rashidian et al., 2005; Rideout et al., 2003; Wang et al., 2002; Zhang et al., 2004). Therefore, whether/how other Prx members are regulated by Cdk members will be of further interest.

In summary, we have uncovered an important mechanism by which calpain-mediated Cdk5 activation regulates DAergic neurodegeneration in an MPTP model of PD via downregulation of Prx2 peroxidase activity. We propose that this loss significantly enhances the ROS environment and leads to DAergic neuron loss (Figure 8). This central pathway in addition to other pathways mediated by additional calpain or Cdk5 targets ultimately lead to nigral degeneration in response to MPTP (Figure 8). These findings are particularly relevant to human PD since both deregulated Cdk5 and increased ROS have been shown in the human PD condition (Jenner, 1998; Nakamura et al., 1997; Przedborski, 2005). Furthermore, we presently, show that phosphorylated Prx2 is increased in human PD patients and that Prx2 phosphorylation is also modified by *dj-1*, a known PD gene (Bonifati et al., 2003). How the latter links to Prx2 phosphorylation will be of great interest in future studies. Taken together, our findings suggest that strategies to modulate Prx2 activity serve as beneficial targets for treatment of PD. This is of particular importance since Cdk5 is thought to have normal beneficial roles in neurons (Li et al., 2002) and modulating a relevant downstream target rather than Cdk5 directly may be a better therapeutic strategy with regard to this pathway.

EXPERIMENTAL PROCEDURES

Animals

Eight-week-old male C57BL/6 mice (22–28 g; Charles River Laboratories, USA) were used for MPTP experiments. All animal experiments conformed to the guidelines set forth by the Canadian Council for the Use and Care of Animals in Research (CCAC) and the Canadian Institutes for Health Research (CIHR) and had approval from the University of Ottawa Animal Care Committee.

Antibodies

The following antibodies were utilized: Tyrosine hydroxylase (TH) (Immunostar, USA), C-8 for Cdk5 (Santa Cruz, USA), C-19 for p35 (Santa Cruz, USA), β-Actin (monoclonal, Sigma, Canada), Prx2 (monoclonal, Abcam, UK), and Alex-labeled secondary antibodies (Invitrogen, Canada). Prx2 and phospho-Prx2T89 polyclonal antibodies were generated and initially purified from rabbit using standard protocols from Biogenes (Berlin, Germany) by immunization with carrier protein-conjugated phosphopeptide, LAWINpTPRKEGGLG. The phospho-Prx2 antibody p-T89 was obtained by first purifying the serum using the phosphorylated peptide. The pan-Prx2 antibody was obtained using the nonphosphorylated peptide. The phospho-specific antibody was further purified by adsorbing onto bacterially expressed and purified GST-Prx2 to remove any remaining crossreactivity to nonphosphorylated Prx2. MAP2 was obtained from Santa Cruz (H-300; 1:300).

Isolation of p35-Binding Proteins

The assay was carried out as previously described (Qu et al., 2002).

Mass Spectrometry

The specific bands on GST-p10 lane were subjected for protein identification by a tandem mass spectrometry as previously described (Shevchenko et al., 1996; Wilm et al., 1996).

Fusion Proteins

All GST and His fusion proteins were expressed in *E. coli* and affinity purified using GSH-beads and Ni-NTA Agarose (QIAGEN Inc, Canada) as per manufacturer's instruction.

In Vitro Binding Assay

The binding assay was performed as previously described (Qu et al., 2002).

Yeast Two-Hybrid

Plasmid construction: Plasmids were constructed using standard subcloning procedures. Briefly, an NcoI/SalI digest of p10 and an NcoI/BamHI digest of Prx2 were subcloned into NcoI/XhoI-digested pAS2-1 (Clontech, Canada) and Nco/BamHI-digested pACT2 (Clontech), respectively. Yeast two-hybrid screening (Clontech) pAS2-p10 and pACT2-Prx were transformed into Y187 and AH109 strains by the LiAc method (Ito et al., 1993) and plated onto SD-Trp^- and SD-Leu^- plates, respectively, as previously described (Mao et al., 2004). Plates were incubated for 5 days at 30°C. Resultant colonies were mated and selected on SD-$Leu^-Trp^-His^-$ for 3–7 days at 30°C.

Immunoprecipitation

Samples were harvested in lysis buffer (50 mM Tris-HCl [pH 7.4], 100 mM NaCl, 1 mM EDTA, 1 mM DTT, and 0.2% Triton X-100) supplemented with protease inhibitors. Immunoprecipitations (IPs) were performed through incubation of antibodies with lysates overnight followed by incubation with anti-rabbit or anti-mouse Ig IP beads (eBiosciences, USA) at 4°C for 1 hr. The beads were washed three times by lysis buffer without protease inhibitors.

Neuronal Cultures

The primary culture of mouse cortical neurons was carried out as described previously (Fortin et al., 2001; Xiang et al., 1996). Alternatively, for midbrain neuronal cultures, the whole midbrain, without meninges and blood vessels, was collected from embryos aged 13.5 days gestation and processed as above and as similarly described (Liu et al., 2000). Cultures were subject to 20 μM MPP^+ or rotenone (as indicated in text). In select experiments, neurons were also pretreated with the calpain inhibitor PD150606 (Calbiochem, Canada), the Cdk inhibitor,

roscovitine (Sigma, Canada), or lithium (BDH, Canada) for 3 hr and then cotreated with 20 μM MPP^+. For survival using $p35^{-/-}$ neurons, littermate controls, or siRNA knockdowns, the MTT assay was utilized as per manufacturer's instruction (Sigma, Canada). For transfection or infection cultures, the alternative strategy described below was utilized since only a small percentage of the neurons in culture were targeted.

ROS Imaging

Cortical neurons were incubated with 10 μM DCF for 20 min at 37°C and washed three times with NB medium. The fluorescence signal of oxidized DCF was observed by an inverted fluorescent microscope equipped with a 100 W xenon lamp and filter (for oxidized DCF, excitation = 488 nm and emission = 510 nm). At least four random fields were quantified for DCF-positive cells and/or average intensity by image analyses.

Infection and Calcium Phosphate Transfection of Cultured Neurons

Cortical neurons were mixed with adenovirus at MOI of 50 prior to plating and were then immediately seeded to 24-well plates and cultured for three days as previously described (Aleyasin et al., 2004; O'Hare et al., 2005; Zhang et al., 2006). The cultures were exposed to MPP^+ for 48 hr. Cultures were then fixed and stained with Hoechst 33258 (0.5 ng/ml) and neuronal survival was evaluated by assessing nuclear integrity of GFP-positive neurons as previously described (Aleyasin et al., 2004). For transfection, 3 days after plating cortical neurons were transiently transfected using a modified calcium phosphate precipitation protocol (Xia et al., 1996; Zhang et al., 2006). In brief, neurons were transfected with 1 μg of total plasmid DNA (0.75 μg of plasmid DNA and 0.25 μg of pEGFP as a reporter) purified using an EndoFree Plasmid Maxi kit (QIAGEN, Inc, Canada). Twenty-four hours post-transfection, neurons were treated with 20 μM MPP^+ (48 hr) and were fixed in 4% paraformaldehyde (containing 0.2% picric acid in 0.1M phosphate buffer [pH 6.9]) and evaluated as described above. Alternatively, neurons were transfected with double-stranded short-interfering RNA (siRNA) to Prx2 or CY3-labeled control duplex (60 pmol siRNA/24-well) as previously described (Zhang et al., 2006). We have observed that targeting of duplexes to neurons is much more efficient than that of plasmids and have used this procedure previously (Aleyasin et al., 2004; Zhang et al., 2006). The Prx2 duplexes (s1: GCUUUCG GACUACAGAGGG, s2: GGGAUUCUUUAAGAGCUCU, s3: CCAAAU AAUUACUAGGCCU) along with a Cy3-labeled control duplex were obtained from Ambion (Austin, TX, USA). Forty-eight hours post-transfection, neurons were treated with MPP^+ (20 μM). At appropriate times, the cells were assayed for survival by MTT method (48 hr) or ROS as described above. Alternatively, cultures were analyzed by Western blot analyses for Prx2 levels (24 hr).

Peroxidase Activity Assay

Peroxidase activity was carried out by measurement of the consumption of NADPH (Fisher) which was mediated by Trx (Sigma, Canada) and Trx reductase (Sigma, Canada) at 30°C for 10 min for bacterially expressed proteins and 1 hr for precipitated proteins from cultured neurons or the SNc. In brief, 0.5 μg of bacterially expressed proteins or the precipitated proteins was incubated with 5 μM Trx, 1 μM Trx reductase, and 100 μM NADPH in HEPES (pH 7.5). The reaction was initiated by the addition of H_2O_2 at a final concentration of 0.2 mM. The consumption of NADPH was measured at 340 nm by spectrophotometer.

MPTP Administration

Mice received one intraperitoneal (i.p.) injection of MPTP·HCl per day (25 mg of free base per kg of body weight per injection; Sigma) for 3 or 5 consecutive days (Crocker et al., 2001; Kalia et al., 2004; Kim et al., 2005; Smith et al., 2003); control mice received an equivalent volume of 0.9% saline. Brains were extracted at indicated times and either perfused for immunohistochemical analyses or quickly removed and dissected for biochemical analyses.

Intrastriatal Administration of Adenoviruses

The adenoviruses expressing Prx2, Prx2T89A, and Prx2T89E were engineered using pAdEasy system as previously described (Sedarous et al., 2003). We and others have previously shown that adenoviruses can target the SNc from the striatum by retrograde transport (Crocker et al., 2001, 2003; Kalia et al., 2004; Kim et al., 2005; Smith et al., 2003). Each adenovirus was injected directly into the striatum of animals 7 days before initiation of MPTP treatment (as described above). A GFP-containing construct was used as a control. A single unilateral injection of each virus (2 μl, 1×10^7 particles per μl) was delivered to the right striatum (0.5 mm rostral, 2.2 mm right of bregma, and 3.4 mm below the skull surface). Each adenovirus injection was given at a constant rate (0.5 μl/min) by using a syringe pump system. Brains were extracted for immunohistochemistry and western blot analysis 14 days after the first MPTP treatment. Double-labeling experiments with GFP (present in all viral vectors) and TH indicated that the majority of SNc TH-positive neurons at the level of the medial terminal nucleus were also GFP positive for all viruses injected (GFP control, Prx2, Prx2T89A, and Prx2T89E).

Immunocytochemistry

Mice were perfused transcardially and brains were fixed in paraformaldehyde and cryoprotected as previously described (Crocker et al., 2003). Serial coronal sections (14 μm thickness) of the ventral midbrain were collected as free-floating sections in 0.01 M PBS/0.02% sodium azide or collected on slides. Sections were then incubated in primary antibody (to TH, 1:10,000; p-T89, 1:100, in 0.3% Triton X-100/0.01 M PBS) for 24 hr at 4°C. For TH staining on floating sections, slices were then incubated with biotinylated secondary antibody and streptavidin horseradish peroxidase-conjugated tertiary antibody and visualized by using a 3,3′-diaminobenzidine/glucose oxidase reaction as previously described (Crocker et al., 2003). To examine the distribution of phosphorylated Prx2 in DAergic neurons, a double-labeling immunofluorescence approach was used. After incubation with the specific primary antibody at 4°C, immunolabeling was visualized by using either Alex-488-conjugated anti-rabbit IgG (1:2000) or Alex-594-conjugated anti-mouse IgG (1:2000).

Quantification of DAergic Neuron Loss

The number of DAergic (TH-positive) neurons was only counted from the sections in the region containing the medial terminal nucleus (MTN) because this region has been previously shown to be expressed at the highest level of virus-mediated gene expression after intrastriatal infection (Crocker et al., 2001). We also used the MTN as a landmark to evaluate consistent levels of the SNc. Neurons ipsilateral and contralateral to the viral injection were assessed as described above in at least three sections per animal. The number of neurons from ipsilateral or contralateral was then counted as previously described. Alternatively, cresyl violet staining was performed to validate determination of nigral counts as previously described (Crocker et al., 2003).

Western Blot Analysis for the SNc

The western blot assay was performed as previously described (Smith et al., 2003). In brief, 50 μg of protein was analyzed by SDS-PAGE using antibodies to phospho-Prx2T89, Prx2, and β-actin.

Human Brain Samples

Paraffin-embedded blocks of postmortem human midbrain were collected from the Ottawa Hospital Department of Pathology. Autopsies were performed according to the policies and procedures of The Ottawa Hospital with consent from the next-of-kin. The tissue was deparaffinized in xylene and subjected to citrate antigen retrieval (Martins et al., 1999) prior to DAB staining. Diagnoses of PD were made based on medical histories and postmortem confirmation (J.M.W.).

The mean average age for PD (n = 5; 4 males and 1 female) and control patients (n = 6; 6 males) was 72.6 ± 3.7 and 72.5 ± 3.6, respectively, and showed no significance ($p < 0.986$). Postmortem intervals for PD and control samples did not differ significantly ($p < 0.3$).

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/55/1/37/DC1/.

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