

Understanding Parkinson's Disease: Mechanisms of Action of DJ-1

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Abstract

Parkinson's disease (PD) is the most common movement neurodegenerative disease affecting approximately 1% of the population over 60. Though originally thought to be sporadic in nature, a genetic component is increasingly being linked to the disease. Of these genes, mutations in DJ-1 (*PARK7*) cause early onset autosomal recessive PD. Initial workup of the DJ-1 protein has suggested that it may act in the cell by combatting oxidative stress though the mechanism by which it does so is unclear. Thus, though much work has attempted to elucidate a function at the biochemical, cellular and organismal level, the overt physiological role of DJ-1 remains elusive. In this dissertation, we explore the mechanisms through which DJ-1 confers neuroprotection, particularly in the case of oxidative stress insult. We demonstrate that DJ-1 acts through the pro-survival protein AKT to accomplish its neuroprotective function. Moreover, we note that DJ-1 likely serves its role as an antioxidant through the NRF2 master antioxidant regulator pathway a pathway that is, itself, likely to be regulated by AKT. Together, our results demonstrate that neuroprotection by DJ-1 is done through a signaling pathway involving both AKT and NRF2 and that disruption of the former in PD likely results in abolishing this signaling pathway. Finally, to generate a better animal model of PD, we demonstrate that backcrossing DJ-1 null mice - which originally did not demonstrate any gross histopathological or behavioral phenotypes - display unilateral dopaminergic degeneration that progresses to bilateral degeneration with aging, a feature reminiscent of classical PD progression. Collectively, this thesis takes a two-sided approach to address the biochemical and physiological functions of DJ-1 within the cell and the mouse in hopes of elucidating mechanisms of neuronal death to devise better translational therapies.

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List of Abbreviations:

-/-	Gene knock-out
6-OHDA	6-HydroxyDopamine
AAV	Adeno Associated Virus
AKT	Also, Protein Kinase B (PKB), Ak strain with Thymoma
AofO	Age of Onset
ARE	Antioxidant Response Element
ATP	Adenosine TriPhosphate
BAC	Bacterial Artificial Chromosome
COMT	Catechol- <i>O</i> -methyltransferase
Ctx	Cortex
CUL3	Cullin 3
CV	Cresyl Violet
DA	DopAmine
DAT	DopAmine Transporter
DBS	Deep Brain Stimulation
DLB	Dementia with Lewy Bodies
DM	Dorsal Motor nucleus
GAD	Glutamic Acid Decarboxylase
GBA (GCase)	GlucocerBrosidAse
GDNF	Glial cell-Derived Neurotrophic Factor
GI	GastroIntestinal

GPe	<i>Globus Pallidus pars externa</i>
GPi	<i>Globus Pallidus pars interna</i>
GS	Glutathione Synthase
GWAS	Genome Wide Association Study
HA (tag)	Hemagglutinin
HC	Higher order sensory association areas and prefrontal fields in Cortex
HO-1	Heme Oxygenase-1
IP	IntraPeritoneal or ImmunoPrecipitation
KEAP1	Kelch-like ECH-Associated Protein 1 (a.k.a. iNRF2)
KO	Knock Out
L-DOPA	<i>Levo</i> -3,4-dihydroxyphenylalanine
LB	Lewy Body
LC (or CO)	Locus Ceruleus
LRRK2	Leucine Rich Repeat Kinase 2
MAO-B	MonoAmine Oxidase - B
MC	MesoCortex
MEF	Murine Embryonic Fibroblast
miRNA	micro RNA (RiboNucleic Acid)
MPP ⁺	1-methyl-4-phenylpyridinium ion
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MTN	Medial Terminal Nucleus
mTOR	mammalian Target Of Rapamycin
NQO1	NAD(P)H dehydrogenase, Quinone 1

NRF2	NFE2L2, Nuclear Factor (Erythroid-derived 2)-Like 2
PBS	Phosphate Buffered Saline
PD	Parkinson's Disease
PDK	PDPK1 or PDK1, 3-Phosphoinositide Dependent protein Kinase-1
PET	Position Emission Tomography
PFA	ParaFormAldehyde
PH (domain)	Pleckstrin Homology
PI3K	Phosphatidylinositol 3-Kinase
PINK1	PTEN INDuced Kinase 1
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PTEN	Phosphatase and TENsin homolog
REM	Rapid Eye Movement
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SGZ	SubGranular Zone
SNc (or SN)	<i>Substantia Nigra pars compacta</i>
SPECT	Single Photon Emission Computed Tomography
STN	SubThalamic Nucleus
SVZ	SubVentricular Zone
<i>t</i> BHQ	<i>tert</i> -Butylhydroquinone
TH	Tyrosine Hydroxylase
UCP	UnCoupling Protein

UPDRS	Unified Parkinson's Disease Rating Scale
UPR	Unfolded Protein Response
UPS	Ubiquitin Proteasome System
VA/VL	Ventral Anterior and Ventral Lateral nuclei (of the Thalamus)
VTa	Ventral Tegmental Area
WT	wild-type (also, +/+)
γ GCS	γ -GlutamylCysteine Synthetase

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List of Manuscripts

- I. Aleyasin H*, **Rousseaux M***, Marcogliese PC, Irrcher I, Parsanejad M, Kim RH, Rizzu P, Callaghan SM, Slack RS, Mak TW, Park DS. DJ-1 Protects the Nigrostriatal Axis from the Neurotoxin MPTP by Modulation of the AKT Pathway. ***Proc Natl Acad Sci USA***, 2010 Feb 16; 107(7): 3186-3191.

(* = Authors contributed equally) *Cited as “Must Read” by F1000 Neuroscience (Thomas B, Beal M: 2010. F1000.com/1927965#eval1482064)*
- II. **Rousseaux M**, Don-Carolis K, Marcogliese PC, Qu D, Aleyasin H, Mak TW, Murphy TH, Ting JPY, Slack RS, Park DS, In Vivo Neuroprotection by DJ-1 is Governed by its Regulation of the Master Antioxidant Regulator NRF2, ***In Preparation.***
- III. **Rousseaux M**, Marcogliese PC, Qu D, Kim RH, Mak TW, Slack RS, Lagace DC, Park DS, Unilateral Dopaminergic Neuron Loss in a Genetic Model of Parkinson’s Disease, ***In Revision: Proc Natl Acad Sci USA.***

Appended Articles

- I. Qu D, Rashidian J, Mount MP, Aleyasin H, Parsanejad M, Lira A, Haque E, Zhang Y, Callaghan S, Daigle M, **Rousseaux M**, Slack RS, Albert PR, Vincent I, Woulfe JM, Park DS. Role of Cdk5-Mediated Phosphorylation of Prx2 in MPTP Toxicity and Parkinson's Disease. ***Neuron***. 2007 Jul 5; 55(1): 37-52.

- II. Aleyasin H, **Rousseaux M**, Phillips M, Kim R, Bland R, Callaghan S, Slack RS, During M, Mak TW, Park DS. The Parkinsons disease gene DJ-1 is also a key regulator of stroke induced damage. *Proc Natl Acad Sci USA* 2007 Nov 20; 104(47): 18748-53
- III. Rashidian J, **Rousseaux M**, Venderova K, Qu D, Callaghan SM, Phillips M, Bland RJ, During MJ, Mao Z, Slack RS, and Park DS. (2008) Essential role of cytoplasmic cdk5 and Prx2 in multiple ischemic injury models, in vivo. *J Neurosci*, Oct 7; 29(40):12497-505.
- IV. Zhang Y, Parsanejad M, Huang E, Qu D, Aleyasin H, **Rousseaux M**, Rodriguez-Gonzalez Y, Cregan SP, Slack RS, Park DS. Pim-1 kinase as activator of the cell cycle pathway in neuronal death induced by DNA damage. *J Neurochem.*, Jan;112(2):497-510
- V. Huang E, Qu D, Venderova K, Zhang Y, Haque ME, **Rousseaux M**, Tandon A, Slack RS, Kim RH, Mak TW, Woulfe JM, Park DS. Apurinic/aprimidinic Endonuclease 1 (APE1): a critical target of Cdk5 in neuronal death, *Nat. Cell. Biol.* 2010 Jun;12(6):563-71
- VI. Irrcher I, Aleyasin H, Seifert E, Hewitt SJ, Chhabra S, Phillips M, Lutz AK, Bevilacqua L, **Rousseaux M**, Jahani-Asl A, Callaghan S, McLaurin JG, Winklhofer KF, Rizzu P, Rippstein P, Kim RH, Fon EA, Slack RS, Harper ME, McBride HM, Mak TW, Park DS. Loss of the Parkinson's Disease-linked gene DJ-1 perturbs mitochondrial dynamics under steady state conditions. *Hum Mol Genet.* 2010 Oct 1; 19(19):3734-46.

Thesis format

According to the Department of Cellular and Molecular Medicine, and Neuroscience at the Faculty of Medicine of the University of Ottawa, this thesis is written as a general introduction, reviewing the literature surrounding the thesis topic followed by a collection of manuscripts. The thesis is then discussed in the final chapter, is related to other findings in the field and is opened to interpretation.

Chapter one, the general introduction, reviews the current body of knowledge surrounding Parkinson's disease (PD) research. It further provides background knowledge and rationale for chapters two, three and four – the manuscripts describing the doctoral work. It includes sections on the aetiology and pathology of PD, current knowledge underlying various genetic and toxin forms of the disease and cellular pathways which relate to the pathogenesis of the disease.

Chapter two is entitled: “DJ-1 Protects the Nigrostriatal Axis from the Neurotoxin MPTP by Modulation of the AKT Pathway”. This work was published in *Proceedings of the National Academy of Science of the United States of America* in 2009 and is included here exactly as published.

Chapter three is entitled: “In Vivo Neuroprotection by DJ-1 is Governed by its Regulation of the Master Antioxidant Regulator NRF2”. This manuscript is in preparation for submission and peer-review.

Chapter four is entitled: “Unilateral Dopaminergic Neuron Loss in a Genetic Model of Parkinson's Disease.” This manuscript is in revision in *Proceedings of the National Academy*

of Science of the United States of America and is included here exactly as submitted.

Chapter five collectively summarizes the data presented in chapters two, three and four and interprets the findings. Furthermore, a discussion on the current directions that are being undertaken with regards to each project is presented.

Chapter 1:

General Introduction

1.1 – PD etiology

Parkinson's disease (PD) is the most common neurodegenerative movement disorder affecting approximately 1 million North Americans. James Parkinson first described PD almost 200 years ago as a shaking palsy (or *paralysis agitans*) (Parkinson, 1817) and since has been characterized as a complex multifactorial disease often seen as a mixed clinical phenotype. Therefore, PD is officially diagnosed at the time of autopsy although an experienced neurologist can detect PD in the clinic in over 90% of cases (Hughes et al., 2002). It has been difficult to target effective therapies due to the mixed clinical and pathological nature of the disease and its unclear genesis, thus current treatments consist of symptomatic relief.

1.1.1 – Affected population

Aging is the principle risk factor for Parkinson's disease (Collier et al., 2011). In developed countries such as Canada and the United States where the healthy life expectancies are 72 and 70 years respectively (Mathers et al., 2001), the prevalence of PD is approximately 300 per 100,000 individuals (Rajput and Birdi, 1997). However, in developing countries such as Tanzania and Bolivia where the healthy life expectancies are 36 and 53 years respectively (Mathers et al., 2001), the prevalence of PD is approximately 20 and 50 per 100,000 individuals (Dotchin et al., 2011; Nicoletti et al., 2003). Therefore, its prevalence is highest in countries where the aging population is increasing. In these affected populations, males are more affected than females (Baldereschi et al., 2000). In addition, there is growing evidence suggesting that both environment and genetics play important roles in the pathogenesis of disease.

1.1.1.i – Sporadic PD

The majority of PD is thought to be idiopathic (of unknown cause) in origin. Several studies over the past few decades have permitted the field to appreciate various environmental susceptibility factors for the disease and test them in a laboratory setting. Nevertheless, these studies are correlative and it remains difficult to establish a direct causal link to PD. These include, but are not limited to, age, exposure to heavy metals (such as manganese and iron, particularly in miners), pesticide exposure (namely Rotenone, Paraquat and Maneb in farmers) and head trauma.

Collectively, understanding of how these factors interact with an individual's genetic make-up have thus far yielded a better understanding of the etiology of PD, although pathogenesis of PD is still far from being fully understood. Taken together, environmental factors likely contribute to the pathogenesis of PD and, therefore, have been used frequently in a laboratory setting because of their easy manipulation and globally reproducible results (see section 1.6 – pre-clinical models of Parkinson's disease).

1.1.1.ii – Familial PD

Although PD was originally thought to be entirely sporadic in origin, in the late 20th century, a seminal study indicated a familial linkage for the disease (mapped to chromosomal location 4q21-23) (Polymeropoulos et al., 1996). A year later, the first PD gene, α - synuclein was identified (Polymeropoulos et al., 1997). Since then, several PD genes have been linked to autosomal dominant and autosomal recessive traits. Understanding of the function of these genes within the cell and, more importantly, the effect that their perturbation poses on the cell as well as the organism, is potentially important since it could yield better drug targets through better comprehension of the disease pathogenesis.

Furthermore, since the advent of next generation sequencing and high-throughput genome-wide studies, an increasing number of genetic variants have been postulated to confer susceptibility to the disease (Hardy, 2010; Peeraully and Tan, 2012). These include genes previously identified to be linked to PD such as LRRK2 and synuclein but also have included numerous previously unidentified protein variants (Hardy, 2010; Pihlstrom and Toft, 2011; Tsuji, 2010). An example of one of these that has been garnering attention is the Glucocerebrosidase (GBA) gene, homozygous mutations of which cause Gaucher's disease (Jmoudiak and Futerman, 2005; Tsuji et al., 1987). This and other PD-associated genes are alluded to in section 1.5.3. With this broad technical arsenal, the research field can attempt to combine both familial PD genes and PD-associated genes with the sporadic components (environmental exposure to toxicants) with the goal of recapitulating drug-targetable, clinically relevant models (Gao and Hong, 2011).

1.2 – Clinical manifestations

Clinical presentations of PD vary greatly according to the age of the patient, disease stage and presence of other diseases compounding the complexity of the disease. Though predominantly characterized as a disease with motor phenotypes, PD is composed of multiple non-motor features that have great impact on the progression and management of the disease.

1.2.1 – Motor phenotypes

The principal motor components of PD can be subdivided into 4 groups: bradykinesia (difficulty in initiating movements), postural instability, resting tremor and slowness of movement. The Unified Parkinson's Disease Rating Scale (UPDRS) is a common evaluation

method to quantify these clinical presentations. This scale permits consistency between neurologist evaluations and may be used in clinical trials to monitor the efficacy of a treatment. Together, these features are thought to be the product of a loss of dopaminergic neurons in the *Substantia Nigra pars compacta* (SNc) and a resulting disruption in basal ganglia circuitry. The correlates between SNc neuron loss and motor symptom progression can be indirectly evaluated by imaging techniques such as Position Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT). This pathway and associated motor phenotypes are further discussed in section 1.3.1.

1.2.2 – Non-motor phenotypes

In addition to its principal motor phenotypes, PD patients exhibit marked alterations in non-motor systems. Depression is a common feature in PD patients, affecting 30-40% of the PD population (Blonder and Slevin, 2011). Though this may be seen as a disease in and of itself, it is interesting to note that this “secondary depression” is much more treatable than its primary counterpart (Barone et al., 2010; Jefferson, 2011). In this same broad category, one also finds apathy and anxiety as emotional symptoms of the disease (Starkstein et al., 1992). Disruption in the latter symptoms, although not as frequent as depression, may negatively impact cognitive function more than emotion itself thereby making the depression/apathy combination seen in PD patients a difficult therapeutic target (Blonder and Slevin, 2011; Oguru et al., 2010).

In late stage PD, a higher incidence of dementia is observed. In fact, there is a six times increased chance for an individual suffering from PD to develop dementia versus an unaffected individual (Aarsland et al., 2001). In addition, there appears to be a correlation between disease duration and onset of cognitive decline (Aarsland et al., 2004; Aarsland et

al., 2011). This correlation may be explained in part by the progression of the synucleopathy to more rostral parts of the brain according to the Braak hypothesis (see 1.3.1.v for further details).

Another clinical presentation in PD patients is sleep disturbance, due to disruption of their rapid eye movement (REM) sleep patterns, possibly as a result of altered muscle tone or noradrenergic dysfunction at the level of the *locus coeruleus* (Askenasy and Yahr, 1985; Larsen and Tandberg, 2001; Mouret, 1975; Stocchi et al., 1998; Tan et al., 1996). In addition, a key side effect of dopamine replacement therapy is vivid dreams and hallucinations due to overstimulation of certain dopaminergic systems. This finding is further discussed in section 1.4.1.

Two early symptoms are thought to occur in pre-clinical PD: anosmia (loss of sense of smell, (Doty et al., 1988; Haehner et al., 2011; Hawkes and Shephard, 1993)) and gastrointestinal troubles (principally constipation, (Braak et al., 2006; Kupsky et al., 1987; Qualman et al., 1984; Wakabayashi et al., 1988)). Moreover, anosmia and GI-disturbances have been suggested to be indicators of PD years before disease onset. As a result, it has been hypothesized that a foreign body (toxin or virus) enters the body by these two ports of entry and selectively propagates through the nervous system, selectively targeting catecholaminergic neurons and resulting in the pathogenesis and progression of PD. These aspects are garnering more and more interest as the field is shifting towards more multifactorial approaches and is discussed in section 1.3.1.v.

1.2.3 – Laterality

The unilateral nature of the disease is often overlooked in basic research pertaining to PD (Djaldetti et al., 2006). Moreover, a unilateral-to-bilateral progression is a common

occurrence. Nevertheless, the origin of this lateral phenotype remains elusive though it is clearly important in better diagnosing the early stages of PD. For instance, in the clinic, the Hoehn and Yahr PD staging system begins with unilateral motor symptomology that, at later stages, progresses to a more symmetrical, bilateral disease (Table 1.1).

Table 1.1: Modified Hoehn and Yahr scale (adapted from (Goetz et al., 2004)).

Staging of Parkinson's disease as first described by Hoehn and Yahr (Hoehn and Yahr, 1967) was modified to better depict the progression of the disorder. Ratings of 1 and 1.5 correspond to symptoms of unilateral origin whereas 2 through 5 correspond to bilateral features that progressively result in severe disability and culminate in autonomic dysfunction.

Modified Hoehn and Yahr Scale

- 1.0 Unilateral involvement only.
 - 1.5 Unilateral and axial involvement.
 - 2.0 Bilateral involvement without impairment of balance.
 - 2.5 Mild bilateral disease with recovery on pull test.
 - 3.0 Mild to moderate bilateral disease; some postural instability; physically independent.
 - 4.0 Severe disability; still able to walk or stand unassisted.
 - 5.0 Wheelchair bound or bedridden unless aided.
-

1.3 – Anatomical and cellular pathology in PD

Parkinson's disease can be distinguished from other similar neurodegenerative diseases principally on its pathological features. Its cardinal features are that of specific neurodegeneration in – though not limited to – the *Substantia Nigra pars compacta* (SNc) as well as the appearance of cytoplasmic inclusions termed Lewy Bodies (LB) in the surviving neurons.

1.3.1 – Affected areas

Although principally highlighted as a disorder of the nigro-striatal pathway, PD pathology expands to various other areas of the brain thereby accounting for its multitude of clinical phenotypes.

1.3.1.i – Substantia Nigra Pars Compacta (SNc)

Considered the hallmark in PD pathology, degeneration of the dopaminergic neurons of the SNc in the midbrain result in the principal motor phenotypes observed in PD. In humans, degeneration of SNc neurons is apparent on the gross examination as an absence of black appearance of the structure in the midbrain (*Substantia Nigra* translates into “Black Substance”).

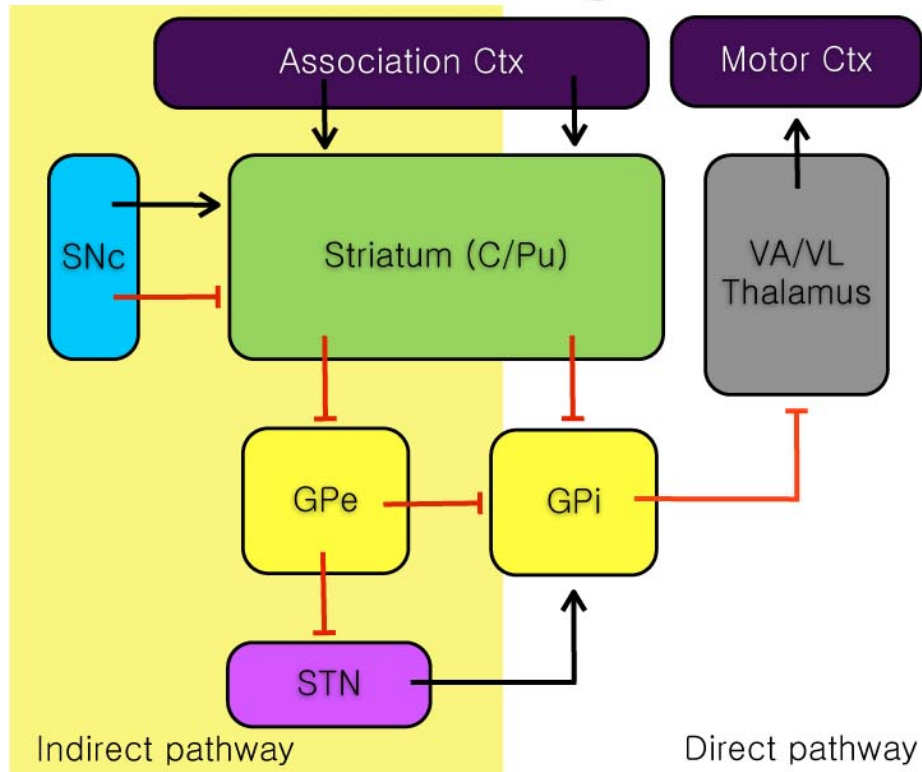
This natural black staining in the SNc results from the presence of neuromelanin (a black pigment) in the cell bodies of dopaminergic neurons of the midbrain. As a result, one can easily confirm the clinical finding of a PD patient using pathological evaluation. The link between SNc degeneration and clinical behavior correlates is due to the disruption of the nigro-striatal pathway in the basal ganglia circuitry (Figure 1.1). In the context of PD, a reduction of innervation to the *striatum* affects both direct and indirect pathways within the

basal ganglia. This reduction in innervation of the nigro-striatal axis – via dopaminergic cell loss in the SNc – has for ultimate result the loss of neural innervation to the motor cortex and a concomitant decrease in movement. Surgical treatments such as Deep Brain Stimulation (DBS) have focused on stimulating and/or delivering genes via viral vectors to nuclei within the basal ganglia downstream of the SNc (i.e. the SubThalamic Nucleus, STN) to bypass this loss and are addressed in further detail in section 1.4.3.

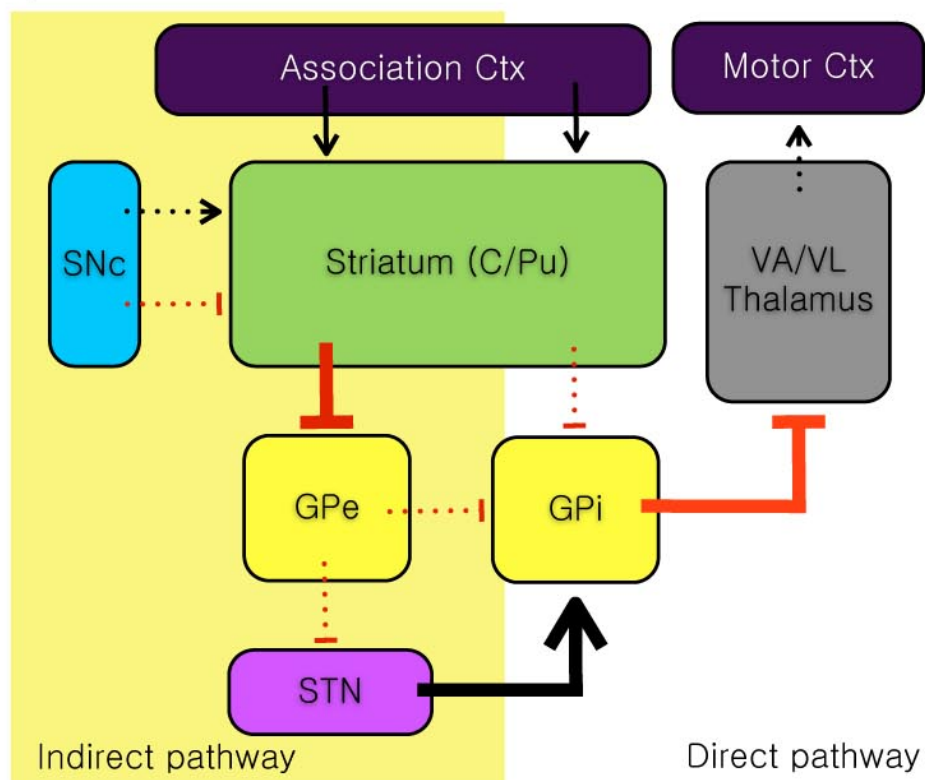
Figure 1.1 Basal ganglia circuitry in the context of Parkinson's disease (Adapted

from (Purves, 2001)). A) Schematic diagram of the basal ganglia circuitry under normal conditions. Briefly, the SNc projects its dopaminergic terminals to the *striatum* where it takes part in the direct (D_1 receptors, activating) and indirect (D_2 receptors, inhibiting) pathways. At this point, the *striatum* (medium spiny neurons mostly) tonically inhibit thalamocortical function through interactions with the internal and external parts of the *Globus Pallidus* (GPi and GPe, respectively) thereby creating a homeostatic activation of the motor cortex (Motor Ctx). Specifically, the indirect pathway consists of projections from the *striatum* to the GPe that will inhibit the SubThalamic Nucleus (STN) from activating the GPi. In contrast, the direct pathway consists of the *striatum* directly inhibiting the GPi to tonically inhibit the ventral anterior and ventral lateral nuclei of the thalamus. B) In PD, neurons of the SNc die and therefore there is decreased striatal innervation. As a result, there is an increase in thalamocortical inhibition (via the GPi) resulting in a decreased in activation in the motor Ctx.

A) Normal functioning



B) Parkinson's disease



A principal question still exists: Why is there specific dopaminergic degeneration at the level of the SNc neurons compared to other areas of the brain? Two studies published in *Nature* may have hinted at an explanation of this. The lab of D. James Surmeier studied the role of oxidant stress – evoked by mitochondrial calcium buffering through L-type calcium channels – in rendering the dopaminergic cells of the SNc more susceptible to death over their dopaminergic counterparts in the Ventral Tegmental Area (VTA) (Chan et al., 2007; Guzman et al., 2009; Guzman et al., 2010). The mechanism by which this may occur is still unclear. However, it was suggested in these studies that the PD gene DJ-1 might play a role in regulating mitochondrial UnCoupling Proteins (UCPs).

Since these pioneering studies, Surmeier's group has further studied the role of L-type calcium channel blockade in neurodegeneration and has shown it to be protective in toxin-induced models of PD (Ilijic et al., 2011). In addition, the (fairly) selective CaV1.3 L-type calcium channel blocker isradipine (Dynacirc CR) has been shown to be well tolerated in PD patients and is therefore in clinical trials as a potential disease-ameliorating drug ((Simuni et al., 2010; Surmeier et al., 2011), Clinical Trials Identifier: NCT00909545).

1.3.1.ii – Locus Coeruleus (LC)

The *Locus coeruleus* (LC) is a nucleus in the pons principally responsible for physiological responses to stress or panic. Interestingly, like the SNc, the LC is pigmented by melanin giving it a blue appearance (hence the Latin name meaning “blue spot”). In brains from *post-mortem* PD patients, one can observe degeneration in the LC. This pathological finding is thought to primarily account for sleep disturbance in PD patients. This finding correlates strongly with the observation that REM sleep and anxiety phenotypes are highly

prevalent in PD patients (McCarter et al., 2012; Richard, 2005). The secondary features of the LC has been suggested to be trophic support to the SNc and therefore their loss may account for increased susceptibility of death to the SNc neurons (Gesi et al., 2000). Finally, disruptions in the synaptic connections sprouting from the LC may also play a part in the postural instability seen in PD (Grimbergen et al., 2009).

1.3.1.iii – Ventral Tegmental Area (VTA)

The Ventral Tegmental Area (VTA) is a dopaminergic cluster of neurons that is adjacent and medial to the SNc. From these dopaminergic neurons stem the mesolimbic and mesocortical pathways that are responsible for reward and motivation systems under physiological conditions. Indeed, this area of the brain is the target of many drugs of abuse including cocaine and amphetamine (Giros et al., 1996; Wise and Bozarth, 1985). In PD patients, however, the VTA can be hyper-activated from dopaminergic precursors and agonists. Therefore, this dopaminergic modification may account for pathological gambling and impulse buying reported in certain PD cases (Vilas et al., 2012). Additionally, in postmortem brains of PD patients, LBs readily accumulate in the cells of the VTA. What is perplexing however is their relatively heightened resistance to death compared to the SNc neurons. This divergence between clusters may be due to the oxidant stress evoked by the autonomous pacemaking in SNc neurons (as alluded to earlier) yet extensive work remains to be done to confirm this. Ultimately, understanding how VTA neurons are resistant to death versus those in the SNc may aid in the development of therapeutics for PD.

1.3.1.iv – Other rostral nuclei of the brain.

Lewy Body structures have also been reported in rostral nuclei in the brain stem such as the dorsal motor nucleus, intermediate reticular zone and raphe nucleus (Muller et al.,

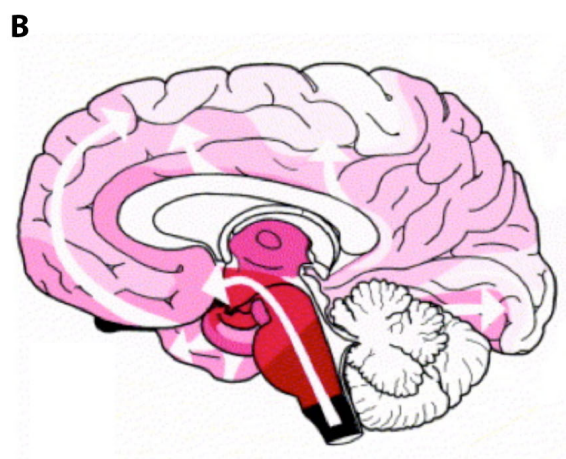
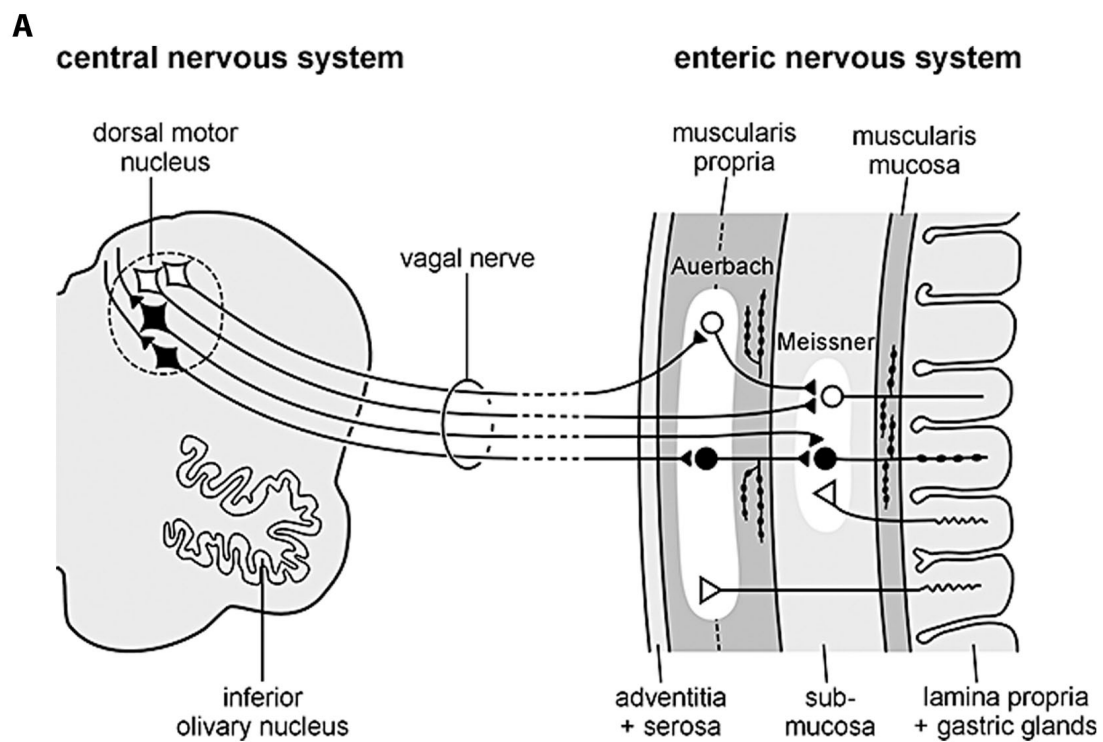
2005). These may each individually have an effect on the clinical presentation of the disease though it has been difficult to tease out specific pathways thus far. Nevertheless, the anatomical and temporal appearance of LBs in PD patients has brought forth a disease progression hypothesis pioneered by Dr. Heiko Braak.

1.3.1.v – Braak hypothesis

The Braak hypothesis stems from a staging system that suggests that the SNc is only affected during mid-stage disease whereas more caudal; components of the brain are affected earlier (Braak et al., 2003a; Braak et al., 2003b). From this staging, Braak and colleagues went on to suggest that PD-pathology may originate through a pathogen (potentially a virus or environmental toxin) at two principal points of entry to the body: the olfactory and the gastro-intestinal (GI) routes. In support of this notion is the findings that over 95% of PD patients exhibit anosmia (loss of the sense of smell) prior to the onset of symptoms (Haehner et al., 2011); and that approximately 50% of PD patients report constipation and general GI disturbances decades before the clinical manifestation of the disease (Braak et al., 2006; Hawkes et al., 2007).

Interestingly, new findings in α -synuclein-centered research suggest that this protein may act to form LBs by propagating from cell to cell in a cell-autonomous fashion – an action much like the prion protein in prion diseases (Angot et al., 2010; Luk et al., 2009; Volpicelli-Daley et al., 2011). Hence, it is plausible that an initial environmental insult or genetic susceptibility may cause a misfolding process in α -synuclein that then accelerates and propagates through time in a rostral fashion leading to LBs not only in the nigro-striatal axis but also often progressing to the cortex resulting in dementia phenotypes (Figure 1.2).

Figure 1.2: Braak hypothesis of pathological staging in PD (adapted from (Braak et al., 2003a; Hawkes et al., 2007)). A) Interactions between the enteric nervous system in the intestinal epithelium and the central nervous system occur at the level of the vagus nerve. This nerve projects to the dorsal motor nucleus and from there, Lewy Body pathology is thought to propagate in a rostral fashion throughout the brain (B). This results in a gradation of pathology severity starting at the Dorsal Motor nucleus (dm) and ending at the frontal cortex where it may contribute to the cognitive decline observed in PD (C). Other notable areas are the *locus COeruleus* (co), the *Substantia Nigra* (sn), anteromedial temporal MesoCortex (mc) and the High order sensory association areas and prefrontal fields in the Cortex (hc).



C

	dm	co	sn	mc	hc	fc
PD-stages	1					
2						
3						
4						
5						
6						

1.3.1 – Cellular responses to protein aggregation

The accumulation of misfolded and aggregated proteins is a common feature of neurodegenerative diseases. In PD, the pathological cytosolic aggregates (LBs) are composed of α -synuclein and ubiquitin amongst other proteins (Spillantini et al., 1997). In a physiological context, the cell has established a multitude of quality control pathways to respond effectively to misfolded or aggregated proteins. For instance, the Unfolded Protein Response (UPR) consists of a system that the cell has in place to dispose of misfolded proteins (Schroder and Kaufman, 2005). To do so, proteins synthesized in the lumen of the endoplasmic reticulum (ER) are constantly surveyed by molecular chaperones that assure their proper folding. Upon irreparable misfolding, the aberrant protein may be transported for degradation by the proteasome through association of these ER-chaperones: a process termed ER-associated degradation (ERAD).

Another cellular system for protein clearance is through the Ubiquitin-Proteasome System (UPS). In this case, the cell can clear excess translated proteins or aberrantly folded proteins. It does so by a selective cascade of ubiquitin conjugating enzymes that signal target proteins to be degraded by the proteasomal machinery.

Moreover, the process of autophagy has been garnering more interest, particularly in a neurodegenerative context. Briefly, autophagy consists of targeting proteins or organelles for their specific degradation via fusion to a lysosome resulting in a recycling of their constituents for the survival of the cell.

In summary, neurons possess numerous quality control mechanisms in response to aberrant protein folding and aggregation. These, collectively, assure the well being of the cell under physiological conditions. However, upon a stress (e.g., oxidative stress or bacterial

infection) or cellular overload of misfolded proteins (e.g., mutated proteins prone to misfolding or increased amount of synthesized proteins), these systems can no longer respond effectively to the demand and this stress imposes its toll on the cell, often resulting in death (Doyle et al., 2011). This balance between pathophysiological states is alluded to throughout this thesis.

1.3.2 – Neuroinflammation and PD

As much as the brain as an organ has evolutionarily isolated itself from the rest of the system through the blood brain barrier, the immune system nevertheless remains functionally intertwined with it. Under physiological conditions, this function serves effectively to clear debris or aggregates when needed or to signal an infection to the rest of the body. However, under pathological conditions, the interplay between nervous system and immune system may result in exacerbation of the cell death phenotype.

For instance, in PD, work from our lab and others have indicated roles for both the innate and adaptive responses of immunity. (Lira et al., 2011; Mount et al., 2007; Orr et al., 2005). In addition, recent attention has been given to PD genes, particularly LRRK2, with regards to modulation of the immune response in the context of neurodegeneration (Gardet et al., 2010; Hakimi et al., 2011; Liu et al., 2011; Moehle et al., 2012). Though the understanding of the interplay between immune and nervous systems in the context of neurodegeneration is still in its infancy, better comprehension of the pathways involved, particularly with PD genes may lead to better symptom treatment by anti-inflammatory agents.

1.3.3 – Reactive oxygen species (ROS), mitochondria and PD

Extensive studies over the past 25 years have attributed a significant role of reactive oxygen species (ROS) and one of their greatest generators, mitochondria, in the pathogenesis of PD. ROS are a natural by-product of various cellular processes, which, upon deregulation, can lead to neuronal death (Murphy et al., 1989; Ratan et al., 1994). One of the largest sources of ROS in the cell is generated by mitochondria whilst performing cellular respiration. As electron transfer progresses to generate a hydrogen ion gradient inside the mitochondrion to subsequently form ATP, superoxide radicals are created which can react with other small molecules to create various reactive oxygen/nitrogen species (ROS/RNS) and can subsequently damage cellular macromolecules (nucleic acids, proteins and lipids).

These oxygen radicals, in low concentrations, seem harmless to the cell and may even serve a physiological function (Chandel et al., 1998; Dada et al., 2003; Hamanaka and Chandel, 2010). However, overproduction of them in a pathological context leads to death via apoptosis (Simon et al., 2000). The latter finding has been suggested to occur during dopaminergic cell loss in PD. This idea stems from a multitude of studies relating both ROS and mitochondrial dysfunction to PD. For example, markers of mitochondrial complex I deficiency have been demonstrated in PD (Blandini et al., 1998; Schapira et al., 1989; Schapira et al., 1990). In addition, accumulation of mitochondrial genome mutations have been demonstrated in these patients (Tanaka et al., 1996). Furthermore, dopaminergic neurotoxins such as MPTP and rotenone – both of which have been highly linked to Parkinsonism – likely confer their toxic effects through direct inhibition of mitochondrial complex I (Lindahl and Oberg, 1961; Mizuno et al., 1987; Oberg, 1961). In addition, as described in section 1.5.2, genes mutated in autosomal recessive PD (if not most forms of

familial PD) seem to serve some mitochondrial role or another by either localizing to the mitochondria to accomplish their function or regulating the clearance of ROS from the cell (Beal, 2007; Lin and Beal, 2006). Finally, there is extensive evidence in cellular and animal models of PD linking ROS to the pathogenesis of disease (Ekstrand et al., 2007; Jenner, 2003; Vila et al., 2008).

Clearly, mitochondria in the context of PD generated much interest and research focus. Nevertheless, treatments based on these findings suggesting that chelation of mitochondrial ROS may lead to a disease-modifying treatment have fallen short at least in the case of the mitochondrial antioxidant MitoQ (Snow et al., 2010). Therefore, better-targeted research to understand these neuroprotective and pathological mechanisms is required to develop better therapeutics.

1.4 – Current therapies

Currently, there are no disease modifying therapies and the preponderance of basic biomedical research focuses on understanding the pathogenesis of PD. Nevertheless, several treatments have been devised over the years to alleviate symptoms and help patients better manage their ailment.

1.4.1 – Dopamine replacement therapy

For over 50 years, dopamine replacement therapy has been at the heart of PD treatments (Cotzias et al., 1968; Fehling, 1966; Sit, 2000). Seen as a gold standard, co-treatment with Levodopa/Carbidopa effectively relieves PD motor symptoms in an efficacious manner with little to no side effects. Its function consists of adding the dopamine pre-cursor Levodopa (L-DOPA) to the remaining dopaminergic neurons. To do so in pill

form, Carbidopa is added to increase the bioavailability of L-DOPA for better entry into the brain by inhibiting its peripheral breakdown.

The advantages of this treatment are clear and although it does not often show adverse effects, high doses may induce dyskinesias (involuntary movements) in PD patients. Furthermore, its shortcomings weigh heavily on the patient. The fact that this drug, and other dopaminergic agonists alike, only treat PD symptoms and do not slow or halt the progression of the disease are the paramount reason for continued basic research in the field. Ultimately, providing the surviving dopaminergic cells of the SNc with L-DOPA can only be effective up to a point where there are not enough dopaminergic neurons left to effectively perform activities of daily living. Another disadvantage of this therapy is that when side effects do appear, they are often poorly and only partly reversible (Rascol and Fabre, 2001).

Similar to direct dopaminergic replacement therapy, COMT inhibitors (prevents dopamine degradation, Entacapone), dopaminergic agonists (Pramipexole and Ropinirole), and MonoAmine Oxidase B (MAO-B inhibitors (Selegiline, Rasagiline) can often effectively treat symptoms. Interestingly, MAO-B inhibitors have been suggested to be neuroprotective in models of PD pre-clinically and may slow the progression of the disease in humans, although this remains controversial (Rascol et al., 2011; Schapira, 2011).

1.4.2 – Physiotherapy

Physiotherapy is an accessible and effective treatment for all stages of Parkinson's disease particularly when it is used in tandem with another treatment, such as dopamine replacement therapy (Speelman et al., 2011). Again, although this treatment is not disease modifying, it may help delay the onset of the need for assisted living and may ultimately provide partial reprieve to the financial burden on health care. Nevertheless, this treatment

option becomes increasingly ineffective as it becomes more difficult to perform the correct physiotherapeutic exercises as the PD patient ages due to complications associated with the disease (i.e. wheelchair bound, autonomic dysfunction, etc.).

1.4.3 – Surgical treatments

Deep Brain Stimulation (DBS) and adeno-associated virus (AAV)-mediated gene delivery are the two principal surgical treatments that have shown promise. The concept underlying deep brain stimulation is to bypass the pacemaking role of the dopaminergic cells in the SNc projecting to the *striatum* and to implant a pacemaking electrode in the SubThalamic Nucleus (STN) to artificially mimic basal ganglia physiological function. Similar to this neurosurgical approach, AAV-mediated gene delivery is principally directed to the STN to modulate its activity within the basal ganglia.

These techniques have proven to be successful at remediating motor symptoms in PD patients with the principal caveat being patient selection. As PD is a disease affecting a large proportion of the elderly, many of these do not qualify for the treatment (i.e., cannot safely undergo surgery with their pre-existing conditions). Thus, from the entire PD population, the number of candidates for this invasive procedure is low. Nevertheless, with a qualifying patient, both STN-DBS and AAV-mediated delivery of Glutamic Acid Decarboxylase (GAD, the enzyme responsible for the production of the neurotransmitter GABA) have shown great results in improving UPDRS scores in PD patients (Kumar et al., 1998; LeWitt et al., 2011; Moro et al., 1999). Nevertheless, though AAV therapeutics seem promising, they are still in their infancy. This idea is underscored by the fact that Amgen halted AAV-GDNF (Glial Derived Neurotrophic Factor) development for safety reasons, raising questions as to the utility of these therapies. Again, as with most other therapies, if effective,

these would simply halt the progression of the disease and may not necessarily provide a full recovery.

1.4.4 – Stem cell therapy

With regards to stem cell therapy, two potential therapeutic gateways exist. The first is mobilization of endogenous stem cell pools in the brain, which has a principal advantage that it is non-invasive and does not rely on immunocompromising the patient; however, it is also riddled with disadvantages. For one, the two well-characterized stem cell pools (SubGranular Zone (SGZ) and SubVentricular Zone (SVZ)) are far from the SNc. Also, these two zones produce limited numbers of stem cells and are slow to generate. In addition, as they are far from the SNc, effectively guiding these cells to the damaged SNc (or other targets of the basal ganglia for that matter) of the PD patient without surgically adding guidance factors is most likely excessively hard to accomplish.

The other therapy consists of inserting exogenous stem cell grafts. This technique has shown to be potentially efficacious but large immune reaction (host rejection) and a chance for oncogenicity from non-specification must be overcome. Recent advances in “personal therapy” may aid in reducing these risks (Caiazzo et al., 2011; Kriks et al., 2011; Pfisterer et al., 2011). Extensive research has been performed in this field, as stem cell therapy is one of the only PD therapies that may lead to full recovery of the SNc cell. Nevertheless, there remains a large gap between the delivery of stem cells to the brain and their subsequent proper integration into the basal ganglia circuitry. Moreover, as specifying these cells them to pacemaking dopaminergic neurons in the proper anatomical and physiological context becomes closer, a question remains: What will prevent these newly formed dopaminergic

neurons from dying? Evidently, additional basic research using effective animal models must be done in order to understand the disease pathogenesis.

1.5 – PD genes

The discovery and introduction of PD genes on the research front has provided much insight into the pathogenesis of PD. Since the discovery of the first PD gene, α -synuclein, many other players have made their appearance, either as a linked gene or an association or risk factor for the disease. As shown in Table 1.2, these genes can be grouped into three principal categories: autosomal dominant PD, autosomal recessive PD and PD associated. In this section, I broadly review the literature on these highly associated PD genes and end with describing the PD gene DJ-1 whose function is the topic of this dissertation.

Table 1.2: Highly associated PD genes. Genes highly linked to autosomal dominant PD (α -synuclein and LRRK2), autosomal recessive PD (Parkin, PINK1 and DJ-1) and the PD-associated gene GBA are presented here. Chromosomal location, mutation types, disease onset and clinical correlates as well as gene function are presented here as a means for comparison. AofO denotes the age of onset of PD. GCase denotes glucocerebrosidase (GBA). Data retrieved as a review of the literature on PubMed.

Inheritance	Gene	Locus	Chromosomal Location	Mutation Types	Clinicopathological Features	Cellular Function
Autosomal dominant	α -synuclein	<i>PARK1/4</i>	4q21	Point mutations, genomic duplications and triplications	AofO: 30-60. High incidence of LBs	Synaptic maintenance, vesicle release
	LRRK2	<i>PARK 8</i>	12q12	Point mutations	AofO: 40-60. LBs variable. Tau present in some cases	Microtubule dynamics, Phosphorylation of cellular substrates, autophagy and mitochondrial dynamics
Autosomal Recessive	Parkin	<i>PARK2</i>	6q25.2-27	Point mutations, exon deletions	AofO: 7-60. Little to no LBs. Tau positive lesions. Nigral degeneration	E3-Ubiquitin Ligase, Unfolded Protein Response. Mitochondrial maintenance.
	PINK1	<i>PARK6</i>	1q35-36	Point mutations, exon deletions	AofO: 13-60. LBs present in SNc but not LC	Phosphorylation of cellular substrates to promote survival. Mitochondrial maintenance and dynamics.
	DJ-1	<i>PARK7</i>	1p36	Point mutations, exon deletions	AofO: 18-40, no pathology described.	Redox homeostasis, chaperone function, anti-apoptotic, oncogene, signal transduction, RNA binding.
Associated	GBA		1q21	Heterozygous point mutations	AofO $\approx$ 55. Pathology similar to idiopathic PD.	GCCase activity. Functionally interacts with α -synuclein.

1.5.1 – Autosomal dominant PD

Similar to sporadic PD, autosomal dominant PD is principally characterized in alterations in α -synuclein and LRRK2 function. In these cases, age of onset and pathology may fairly-closely resemble idiopathic PD (Chartier-Harlin et al., 2004; Di Fonzo et al., 2005; Haugarvoll et al., 2008; Kachergus et al., 2005).

1.5.1.i – α -synuclein (*SNCA*, *PARK1/4*)

Polymeropoulos and colleagues were the first to clearly establish a familial component to PD. In their study, they identified mutations in the gene *SNCA* (*PARK1*) coding for α -synuclein that were directly associated with PD (Polymeropoulos et al., 1997). Later that year, a pathophysiological role of α -synuclein was alluded to when it was identified as the principal component of LB (Spillantini et al., 1997). In this study, the authors showed strong α -synuclein positive staining in the ubiquitin-positive intracytoplasmic inclusions. It has since become widely accepted that α -synuclein aggregation forms an integral part of “typical” PD and many studies still focus on identifying the mechanisms through which α -synuclein oligomerization and subsequent aggregation is induced to better understand disease pathophysiology.

The mechanism of α -synuclein-mediated pathogenicity has been suggested to arise in its pathological accumulation and subsequent aggregation. This is supported by two principal findings. First, PD patients with genomic duplications or triplications of the *PARK1/4* locus demonstrate increased synuclein expression, aggregation and a dose-

dependent increase in disease-onset and severity (Chartier-Harlin et al., 2004; Ibanez et al., 2004; Singleton et al., 2003). Second, mutants of α -synuclein (notably A53T and A30P) have been shown *in vitro* to directly increase its oligomerization and aggregating properties and subsequently resulting in neuronal death (Conway et al., 2000; Petrucelli et al., 2002). Collectively, homeostatic control of α -synuclein levels and folding appears to be an integral process that permits its proper function and prevents neurodegeneration.

α -synuclein also serves an interesting role in bridging distinct neurodegenerative diseases together by providing insight into common pathways. For instance, PD and Dementia with Lewy Bodies (DLB) are two distinct neurodegenerative diseases with different clinical phenotypes yet they maintain a pathological correlate: α -synuclein positive inclusions. Furthermore, α -synuclein has been an interesting protein to study as it may parallel other aggregation-prone proteins such as β -amyloid, huntingtin and the prion protein; their aggregation being somehow directly related to the disease process (Jellinger, 2011).

Although the function of α -synuclein in a pathological context has been widely described, its physiological role remains poorly understood. It had been established that α -synuclein was a pre-synaptic protein with functions in regulating dopaminergic transmission (Abeliovich et al. 2000), yet no clear biochemical evidence ascribed a clear role for the protein in physiological conditions. In 2004, however, Thomas Südhof's group indicated that α -synuclein played a key role in synaptic transmission and interacted with multiple components of pre-synaptic exocytosis machinery (Abeliovich et al., 2000; Burre et al., 2010; Chandra et al., 2004; Chandra et al., 2005).

As increased α -synuclein dosage and mutations likely function in a gain-of-function manner, mutant mice overexpressing various forms of α -synuclein under the control of different promoters have been generated. One powerful model of note was the mouse generated by Masliah and colleagues that expressed human α -synuclein under the control of the PDGF β promoter (Masliah et al., 2000). This mouse exhibited synucleinopathy, dopaminergic dysfunction and motor behavior dysfunction with fairly high correlation to human PD. Since then, multiple models have been generated to recapitulate PD features in mice as well as rats (using viral delivery of α -synuclein) at a more representative level (Giasson et al., 2002; Lo Bianco et al., 2002; van der Putten et al., 2000).

α -synucleinopathy may be transmitted in cell autonomous fashion. Certain researchers have gone so far as suggesting that α -synuclein – and its improper aggregation – may act similar to the prion protein (Braak et al., 2006; Danzer et al., 2009; Desplats et al., 2009; Luk et al., 2009; Volpicelli-Daley et al., 2011). These laboratory findings are substantiated by two clinical findings. First, that fetal stem cell grafts given to PD patients as a therapy not only did not slow the progression of the disease but also upon autopsy, were riddled with LB-like inclusions (Kordower et al., 2008; Li et al., 2008a; Li et al., 2010a). Second, as mentioned earlier in the Braak hypothesis, pre-clinical PD may originate in the enteric plexus and progress in a rostral manner to the midbrain and eventually to the cortex thus accounting for the gastro-intestinal dysfunction preceding most PD cases as well as the eventual dementia affecting most late stage PD patients.

1.5.1.ii – Leucine Rich Repeat Kinase 2 (LRRK2, PARK8)

In 2004, two reports identified families containing individuals with PD having mutations in the *PARK8* locus (Nichols et al., 2005; Paisan-Ruiz et al., 2004; Zimprich et al., 2004). These mutations were noted to cause point mutations in the LRRK2 protein encoded at this locus. Though mutations in the kinase domain of LRRK2 (G2019S, I2020T) seem to be most prevalent in PD patients, mutations have been found in virtually other domains of the protein including the ROC (Ras Of Complex) domain (R1441C), the COR (C-terminal of ROC) domain (Y1699C) as well as the LRR (Leucine Rich Repeat) domain (I1122V) (Mata et al., 2006). Primary cellular workup on human LRRK2 kinase domain mutations suggested that they might be gain-of-function in origin as they increase its kinase function and are inherited in a dominant manner (Gloeckner et al., 2006; West et al., 2005).

Since these initial findings, however, multiple cellular roles have been attributed to LRRK2 and most research has thus been focused on elucidating the cellular function of LRRK2 under pathophysiological conditions. It has been suggested that the kinase activity of LRRK2 mediates its neurotoxicity although the validity of this finding remains unclear (Abdel Messih et al., unpublished data; Saha et al., 2009; West et al., 2005)). One notable finding has been the capacity of LRRK2 to interact with other cellular components. For instance, LRRK2 has been noted to interact with the PD gene Parkin (Gloeckner et al., 2006). In addition, it forms complexes with neurite outgrowth machinery (Gillardon, 2009; Heo et al., 2010; Lee et al., 2010; Lin et al., 2010; MacLeod et al., 2006; Piccoli et al., 2011; Shin et al., 2008), of which some may be involved in the pathogenesis of PD (Gan-Or et al., 2012; Satake et al., 2009; Simon-Sanchez et al., 2009). In addition, recent studies from our laboratory and others have indicated a putative role for LRRK2 in the regulation of

autophagy (Abdel-Messih et al., unpublished data; (Alegre-Abarrategui et al., 2009; Ramonet et al., 2011; Tong et al., 2010; Xiong et al., 2010)) although whether its endogenous function serves a pro-survival or pro-death role remains unclear.

In order to elucidate the organismal function of LRRK2, multiple animal models have been created. *Drosophila* models overexpressing human forms of LRRK2 and its mutants have revealed loss of dopaminergic neuron clusters and concomitant motor behavior defects (Imai et al., 2008; Liu et al., 2008b; Venderova et al., 2009). In addition, in a zebrafish (*Danio Rerio*) where the WD40 domain of LRRK2 was ablated, Sheng and colleagues noted a loss of dopaminergic cells in the diencephalon and resulting locomotor defects (Sheng et al., 2010). Finally, multiple mouse models ranging from LRRK2 null mice to BAC-overexpressor mice have been generated to better elucidate the functionality of LRRK2 – both native and mutated forms – within the organism (Li et al., 2010b; Li et al., 2009; Melrose et al., 2010; Tong et al., 2010). Nevertheless, these animal models – as with most other murine genetic models of PD – have failed to recapitulate progressive dopaminergic degeneration.

Collectively, delving into these murine models has provided more insight into the function of LRRK2 on a more global scale. Work in our laboratory has indicated that LRRK2 may mediate MPTP toxicity and mitochondrial dynamics (Abdel-Messih et al., unpublished results). In addition, expression of LRRK2 accelerated α -synuclein-mediated toxicity in mice (Lin et al., 2009). Moreover, LRRK2 has been suggested to interact with members of a miRNA pathway (Gehrke et al., 2010) and links with the immune system (Gillardon et al., 2012; Hakimi et al., 2011; Liu et al., 2011; Thevenet et al., 2011). The latter

findings have elicited much interest recently given the roles attributed to α -synuclein in its pathological staging from the gut to the neocortex (see Braak hypothesis, 1.3.1.v).

1.5.2 – Autosomal recessive PD

The genes Parkin, PINK1 and DJ-1 not only share quite similar clinical and pathological correlates but they also may serve similar cellular functions. Onset for cases of PD with mutations in these genes is early and, at least for Parkin, does not often display significant LB pathology. Therefore, though the pathogenesis of the PD is unknown, it is likely that autosomal recessive PD is more acute in origin and perhaps principally centered on the mitochondrion rather than a progressive protein folding disorder and must thus be tackled at a different angle with regards to therapeutic intervention (Kitada et al., 2012).

1.5.2.i – Parkin (*PARK2*)

The first evidence for autosomal recessive PD was made evident in 1998, where a point mutation in the *Parkin* gene at the *PARK2* locus resulted in early onset PD in a Japanese family (Kitada et al. 1998). Later that year, Lücking and colleagues noted deletions in the *Parkin* gene in European and North African early-onset PD patients (Lücking et al., 1998). These studies also suggested a role for Parkin as an ubiquitin-E3 ligase as its predicted structure contained a RING finger motif. Mutations in Parkin are spread out throughout the gene and the mutants are generally thought to be loss of E3-ligase regulatory function of the protein (Nuytemans et al., 2010).

Follow-up studies confirmed this regulatory function and noted that it may play a role in clearing neurotoxic proteins (Imai et al., 2000; Trempe et al., 2009; Zhang et al., 2000). These include the Pael receptor (Imai et al., 2001), α -synuclein (Shimura et al., 2001),

synphilin 1 (Chung et al., 2001), mitofusin (Glauser et al., 2011; Ziviani et al., 2010) and PARIS (a PGC1 α inhibitor, (Shin et al., 2011). Parkin also binds CHIP and endophilin-A, factors which have since been identified to aid the former in its clearance activity of toxic substrates (Mengesdorf et al., 2002; Trempe et al., 2009). Several studies have also linked Parkin to other PD genes, either through direct binding or functional interaction, suggesting converging pathways in the pathogenesis of disease (Olzmann et al., 2007; Smith et al., 2005; Xiong et al., 2009).

Parkin has also been associated with cancer where it has been suggested to act as a tumor suppressor (Cesari et al., 2003) and transcriptionally represses p53 (da Costa et al., 2009). In addition, studies have indicated that Parkin E3 ligase activity is highly regulated, either through S-nitrosylation (Chung et al., 2004; Yao et al., 2004), covalent modification by dopamine (LaVoie et al., 2005), sequestration (Kalia et al., 2004; Sato et al., 2006) or transcriptional modulation (Bouman et al., 2011).

Various animal models have been generated since the identification of Parkin as a PD gene, yet have overall fallen short in mimicking the full human phenotype. For instance, in *Drosophila*, Greene and colleagues generated a *Parkin* null fly and noted mitochondrial pathology and muscle degeneration yet seemingly normal dopaminergic neuron clusters (Greene et al., 2003). In addition, a zebrafish morpholino knockdown of *Parkin* resulted in impaired mitochondrial complex I function and dopaminergic cell loss but without any clear motor phenotype (Flinn et al., 2009). Moreover, mice lacking *Parkin* showed no clear nigrostriatal deficiency different from that of wild-type controls bar perhaps minor dopamine release disruption (Kitada et al., 2009a; Perez and Palmiter, 2005). Nevertheless, heightened overexpression of a truncated form of human Parkin (Q311X) in mice developed

neurodegeneration and behavior defects which may be a promising model to study autosomal recessive PD (Lu et al., 2009).

These animal models have provided insight into the potential relationship between Parkin and inflammation, a key facet of neurodegeneration (Frank-Cannon et al., 2008) as well as mitochondrial dynamics and ROS homeostasis. Additionally, the link between Parkin and the PD gene PINK1 in regulating mitochondrial function has been a topic of great interest to some in trying to understand the interplay between these two genes in the context of neurodegeneration (Narendra et al., 2008; Poole et al., 2008).

1.5.2.ii – PINK1, PTEN-Induced Kinase 1 (PARK6)

PINK1 was first identified in 2001 as a gene responsive to the tumor suppressor PTEN (Unoki and Nakamura, 2001). Around the same time, the *PARK6* locus was associated with PD (Valente et al., 2001). These two findings were later merged when a direct link between PINK1 and autosomal recessive early onset PD was generated. In this case, two homozygous mutations (G309D and W437X) were observed in three consanguineous Italian families (Valente et al., 2004). In this same study, PINK1 subcellular localization was noted to be mitochondrial and this associated function has since been the focus of intense research. For instance, in *Drosophila*, it was noted that PINK1 deficient flies exhibit mitochondrial cristae disruption and functionally display muscle and dopaminergic neuron degeneration (Clark et al., 2006; Park et al., 2006; Yang et al., 2006). Interestingly, in all three studies, this phenotype was rescued by Parkin whereas PINK1 could not rescue Parkin deficiency.

More recent findings have attributed a functional role for the PINK1-Parkin genetic interaction with regards to mitochondrial quality control. In essence, PINK1 is believed to recruit Parkin to damaged mitochondria (a potential feature in human PD) where Parkin can

target these defective organelles for a specific degradation by autophagy, a process termed “mitophagy” (Geisler et al., 2010; Lazarou et al., 2012; Narendra et al., 2008; Narendra et al., 2010; Van Humbeeck et al., 2011; Vives-Bauza et al., 2010). Though this mechanistic finding is interesting and plausible, it remains controversial as to whether this process occurs in more physiologically representative settings (Sterky et al., 2011; Van Laar et al., 2011).

1.5.2.iii – DJ-1 (*PARK7*)

DJ-1, similar to PINK1, was first ascribed as having an oncogenic function. It was first discovered as an oncogene that transformed NIH-3T3 cells cooperatively with h-RAS (Nagakubo et al., 1997). Following this discovery, links to androgen receptor function (Wagenfeld et al., 1998), in addition to RNA binding protein function (Hod et al., 1999), were attributed to DJ-1. Though the link between DJ-1 and oxidative stress may have been as early as 2001 (Mitumoto et al., 2001), it was not until 2003 that a genetic linkage to PD was established (Bonifati et al., 2003). This study and other follow up reports have since linked various mutants (both frameshifts, rearrangements and missense mutations) in the *PARK7* locus that collectively appear to result in loss of protein stability and subsequent function of the DJ-1 protein (Corti et al., 2011).

In the quest of obtaining a better murine model for PD, in 2004-2005, DJ-1 knockout mice were generated by our lab and others and characterized as potential models (Goldberg et al., 2005; Kim et al., 2005b). Unexpectedly, no dramatic pathological or behavior phenotypes were observed. Since then, consistent results have been obtained in other animal models (Andres-Mateos et al., 2007; Chen et al., 2005; Manning-Bog et al., 2007). Thus, the major focus in the PD field has been to elucidate the function of DJ-1 within the cell. Indeed, it has been hypothesized to be instrumental to prevent the genesis and progression of this

disease through its oxidative stress-combatting properties (Kahle et al., 2009). This is the subject of the thesis and is further addressed in section 1.7.

1.5.3 – PD associated genes

1.5.3.i – *Glucocerebrosidase (GBA)*

Variants in the *Glucocerebrosidase (GBA)* gene are associated with a high risk of PD. In fact, GBA is one of the strongest associated PD genes. In a recent study, it was observed that heterozygous carriers for point mutations in the *GBA* gene demonstrate a vast increase in PD and estimates suggests that up to 31% (Aharon-Peretz et al., 2004) (average of 15%, (Sidransky et al., 2009)) of sporadic PD cases may be in relation to polymorphisms in the *GBA* gene. This finding is particularly important given that GBA has been functionally associated with α -synuclein in the cell (Cullen et al., 2011; Mazzulli et al., 2011; Sardi et al., 2011). As the relationship between α -synuclein and GBA having being mechanistically uncovered in these studies, future work will likely address the interplay of these two genes within the cell to modulate the neurodegenerative process.

1.5.3.ii – Other PD-associated Genes

Due to the growing advantages of genome-wide studies, an increasing number of genes including HLA-DRB5, BST1, SYT11, LRRK2 and α -synuclein are being associated to PD (International Parkinson Disease Genomics et al., 2011; Zimprich, 2011). The current focus has since been to establish a functional relationship between these identified genes, their pathways in the cell under pathophysiological conditions and their relationship with the large environmental component of the disease. With this knowledge in hand, one can then

create better model systems to ultimately better understand the onset and progression of this disease.

1.6 – Pre-clinical models

1.6.1 – Invertebrate models

Invertebrate models serve as effective tools to understand fundamental processes underlying the death of dopaminergic neurons. Though the same cells and/or neurological circuits may not be present, these model organisms may be used as screening tools to identify players in neurodegeneration eventually leading to their use in more clinically relevant model organisms.

1.6.1.i – *Saccharomyces cerevisiae*

Though not apparent at first, the common budding yeast (*Saccharomyces cerevisiae*) has proven to be an excellent tool to understand the process of neurodegeneration. Specifically, recent studies over the past decade, principally pioneered by Susan Lindquist, have shed insight into a common facet of neurodegeneration: aberrant protein aggregation (Khurana and Lindquist, 2010). As LB formation is a prominent feature of PD, α -synuclein aggregation in this context has been studied in detail. Using yeast as a model, researchers have been able to perform high-throughput studies at a relatively low cost to effectively identify targets that play a role in the neurotoxic aggregation process. For example, a recent study by Gitler and colleagues remarked that α -synuclein could genetically interact with the PD gene *PARK9* (coding for ATP13A2 in humans). In addition, the group described a mechanism through which *PARK9* alleviates manganese-induced toxicity and identified

several other genetic factors involved in the alpha-synuclein aggregation pathway (Gitler et al., 2009).

Though this model provides a tantalizing approach to study protein aggregation, it possesses its own shortcomings. Indeed, the homology of the genetic analog (as with other invertebrate species delineated below) with its human counterpart is very low. In addition, the study was not performed in a neural system but rather in a single cell organism, thus any outcome identified must be further validated in a higher organism.

1.6.1.ii – *Caenorhabditis elegans*

A major contributing factor for the etiology of PD is aging. As aging has been extensively characterized in *Caenorhabditis elegans*, these factors can be used to study the interplay between the aging process and degeneration of dopaminergic neurons. Almost every transgenic model created using a PD-linked gene has elicited degeneration specific to dopaminergic neurons – a feature not so easily obtained in higher organisms (see section 1.6.2). This model provides an effective way to study the multifactorial nature of the disease. In this way, using *C. elegans*, one may effectively monitor the interplay between genes and environment.

For example, a recent study by Benedetto and colleagues showed that manganese-induced dopaminergic neurotoxicity may be specifically exacerbated by extracellular dopamine and mitigated by the SKN-Bli3 pathway (Benedetto et al., 2010). Although relatively little work has been performed using the worm as a model, as the compendium of molecular techniques continues to grow, these organisms will increase their value. The principle disadvantages of this model organism are:

1. As these are lower invertebrates, they are closer to a cellular model than a system in that there is not a vast interconnectivity of networks to study;
2. due to their small size, biochemistry and cell physiology experiments are difficult; and
3. the homology between PD-linked genes is low ($\approx 30\%$), if not absent (as in the case for α -synuclein).

1.6.1.iii – *Drosophila melanogaster*

Unlike the murine models discussed below, it has been much easier to modulate PD-related genes (silencing or overexpressing) in *Drosophila* and generate models that recapitulate certain facets of the disease. For instance, flies overexpressing wild-type and mutant forms of α -synuclein display age-dependent neurodegeneration, appearance of LBs and locomotor defects (Feany and Bender, 2000). This mutant is just one of many *Drosophila* models that have provided elegant models to understand the neurodegenerative process (Hao et al., 2010; Wang et al., 2006; Yang et al., 2005). In addition, due to their short lifespan and easy maintenance, genetic screens have made it possible to determine interaction networks amongst PD genes.

The Schulz lab performed a fly screen to identify proteins that mitigate α -synuclein toxicity. In this study, the mitochondrial chaperone protein TRAP1 was identified as a key modifier of this phenotype (Butler et al., 2012). From this study, one can then turn to a more pre-clinical model to study the novel modifier in a better neurodegenerative context. In another example, our laboratory is currently investigating a suppressor/enhancer screen for modifiers of LRRK2-mediated toxicity. LRRK2-overexpressing mutant flies have previously

been generated in our lab in an effort to establish a readily observable phenotype that may be monitored for change when a genomic interactor is either overexpressed or absent (Venderova et al., 2009). In this screen, one can easily screen the fly eye for changes when a LRRK2-mutant overexpressing fly line is crossed to other genomic deficiency lines. From this screen, several hits have been identified and are currently being pursued in the Park Lab (unpublished data).

Taken together, both worm and fly models of PD have provided great insight into the pathogenesis of the disease. These models effectively recapitulate (at least within the system's means) the disease process and progression and can permit further exploration through genetic and chemical screens. However, they are not without limitations. For one, these models systems are far from having the intricate neural connectivity present in humans. In addition, their short lifespan make it hard to follow disease progression. Finally, on a cellular level, protein homology remains fairly low and sometimes is absent and therefore the translatability between these models and the clinic can be a far reach at times. Therefore, one must move from these elementary models to more pre-clinical models of disease to afford a better translational approach.

1.6.2 – Murine models

Murine models of PD are probably the most widely used tools to understand the pathogenesis of the disease. The use of these models relies on the breadth of genetic tools, that are available and widely used, as well as their effective response to toxins and viral-mediated gene delivery. Importantly, the anatomical, physiological and behavioral correlates between mice/rats and humans are fairly similar and permit an accurate assessment of the disease process as a whole.

1.6.2.i – Toxin models

1.6.2.i.a – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a neurotoxin that specifically targets dopaminergic cells in dopaminergic cell clusters of multiple higher order species including humans and mice. It was first hypothesized to account for Parkinsonism in 1976 when a graduate student in Maryland, upon attempting to synthesize the meperidine analog MPPP, created MPTP as a by-product and self-administered it intravenously. He was then presented to the hospital 3 days later with Parkinsonian features and when later autopsied, a dramatic loss in dopaminergic cells in the SNc was observed (Langston, 1996).

A few years later, seven other cases appeared and were treated by the neurologist J.W. Langston (Langston et al., 1983). The latter suggested that MPTP was the cause of these PD symptoms and surmised that its injection in animals may replicate clinical PD-features and subsequently published several seminal papers highlighting the use of the drug in pre-clinical research (Ballard et al., 1985; Barbeau et al., 1985; Langston et al., 1984). Due to its lipophilic nature, MPTP can cross the blood brain barrier where it will be taken up by astrocytes and broken down to its active ion, MPP^+ , by MAO-B. There, it is secreted and rapidly enters dopaminergic cells via the Dopamine Transporter (DAT). It is interesting to note that though MPP^+ is taken up in many types of dopaminergic cells, it shows fairly selective toxicity to dopaminergic cells of the SNc.

Since this discovery, MPTP has been a chemical used to study the potential pathogenesis of PD; although, as more thoroughly discussed in chapter 3, its role in identifying neuroprotective drug targets pre-clinically has proven to be surprisingly in discordance with its clinical trial correlates (Parkinson Study Group, 2007) and therefore

may not effectively translate to a clinical setting. Taken together, some advantages in the MPTP model are:

1. it produces parkinsonian features in humans;
2. it is relatively non-invasive as it is injected intraperitoneally; and
3. it preferentially kills dopaminergic neurons in the SNc over those of the VTA.

The principle disadvantages are:

1. it does not produce clear LB-like cytoplasmic inclusions,
2. it acts more acutely than the disease itself, and
3. its apparent poor translatability to the clinic.

1.6.2.i.b – 6-hydroxydopamine (6-OHDA)

6-hydroxydopamine (6-OHDA) is a neurotoxin that has long been used (over 40 years) to induce nigro-striatal lesions in mice and rats (Evetts et al., 1970; Ungerstedt, 1968). Alongside MPTP, it has served as the “gold-standard” for pre-clinical models of PD as its advantages consist of:

1. controlled loss of large amount of dopaminergic neurons in SNc resulting in consistent behavior disruptions;
2. 6-OHDA does not cross blood brain barrier so the administration of the drug is low-risk with regards to toxicity to humans;
3. unilateral degeneration allows for an internal control (contralateral side to lesion) for studies as well as closer similarity to early PD (see Laterality of PD, 1.2.3); and
4. versatility of lesion size.

On the other hand, this model comes with its own set of limitations. Its disadvantages include:

1. it is invasive (i.e. brain surgery, a needle tract traversing the entire brain, and directly targeting a foreign substance to the SNc,
2. no clear human pathological correlate, and
3. acute onset of neurodegeneration with no LB formation.

1.6.2.i.c – Rotenone and other pesticides

The link between pesticides and PD was generated many years back though only recently have case-controlled studies come out to declare rotenone as a major risk factor for PD (Odd ratio 5-10 times more likely to develop PD with a single exposure or more (Dhillon et al., 2008; Hancock et al., 2008). Nevertheless, Rotenone was used previously to bridge the link between these loose environmental associations and neurodegeneration in the lab.

Betarbet and colleagues treated rats with continual intravenous rotenone or vehicle control and demonstrated presence of LB inclusions, dopaminergic degeneration and concomitant behavioral defects (Betarbet et al., 2000). In addition, transferrin-dependent iron accumulation has been noted in this model: a feature also present in human PD (Mastroberardino et al., 2009).

The principal advantages of this toxin model are that it:

1. relates to the idiopathic nature of the disease (environmental exposure to toxins and pesticides);
2. can be performed using chronic exposure and administration routes are usually non-invasive (oral, intraperitoneally, food, and osmotic pumps); and
3. produces LB, which is not typically present in the other toxicant models.

In contrast, its main disadvantages are that it:

1. gives rise to mixed results in terms of cell death and behavior correlates;
2. there is mixed pathology that may be more representative of other neurodegenerative diseases rather than PD (i.e. sometimes more Tau than α -synuclein in the brain of treated rats (Hoglinger et al., 2005)); and
3. it is likely toxic to humans.

1.6.2.ii – Viral models

Viral models have been used for the past few years to deliver or to knock-down specific genes in hope of producing more accurate forms of the disease. In this sense, the use of Adeno-Associated Viral vectors (AAV) has proven to be useful in obtaining effective genetic modulation in a relatively short period of time (2-4 weeks). For instance, overexpression of α -synuclein by AAV in the rat SNc evoked dopaminergic neuronal death, striatal denervation, α -synuclein accumulation and pathology as well as behavior defects (Kirik et al., 2002).

In the case of genetic knock-down by AAV, our lab has employed AAVs encoding small hairpin RNAs (shRNAs) for *PINK1* and have observed their impact on neuronal cell survival with and without the neurotoxin MPTP (unpublished data). In contrast, viral-mediated gene delivery has proven to be useful to rescue certain toxin phenotypes (see Chapters 2 and 3), thus intertwining the environmental and genetic duality of PD. In fact, the use of these vectors has proven successful clinically in aiding in surgical treatment of PD. The use of AAVs to deliver glutamic acid decarboxylase (GAD), a precursor of the inhibitory neurotransmitter GABA, to the STN to better control the basal ganglia circuitry in

PD patients (Phase II clinical trials, (Kaplitt et al., 2007; LeWitt et al., 2011)). This feature, with regards to murine models of PD, is better described in chapters 2 and 3.

Taken together, the advantages of viral models consist of:

1. rapid and controlled expression of target gene;
2. genotype-phenotype correlation that better represents human PD when compared to genetic animal models (see 1.6.2.iii); and
3. relative ease in producing mutant construct to address specific questions.

Disadvantages mainly include:

1. invasiveness of delivery (as with 6-OHDA, injection is directly to the SNc);
2. potential for immunological response;
3. acute onset of injury; and
4. genetic dosing not representative of typical PD (i.e. dramatic overexpression as a result of a potent promoter).

1.6.2.iii – Genetic models

As alluded to in the earlier sections, mouse models based on autosomal dominant forms of the disease appear to recapitulate LB and behavioral defects but fail to show degeneration in their SNc. In addition, the murine model systems based on autosomal recessive PD do not show clear or consistent phenotypes and, though their cellular functions are similar and could be redundant, triple knock-out of DJ-1, PINK1 and Parkin results in a mouse almost indistinguishable from wild-type (Kitada et al., 2009b). The field has thus fallen short in terms of properly recapitulating genetic forms of the disease in higher organisms and has relied on acute toxicant models of injury to screen for pre-clinical targets.

Taken together, though there are a plethora of models to choose from, each comes with a host of advantages and disadvantages. Each model typically offers one component of the disease (i.e. Lewy Body-like structures) while not displaying other prevalent facets (i.e. selective dopaminergic cell death or behavior phenotypes). In addition, the progressive onset and the multifactorial etiology of the disease have been understandably difficult to recapitulate given their complex nature. Collectively, one can thus appreciate the need to integrate the observations made in these other models to generate more relevant models of PD. Once these factors are understood, one can invest into larger longitudinal studies. As we can collectively note, a large gap remains between the bench and the bedside with regard to pre-clinical models of PD.

1.7 – DJ-1 functions in response to oxidative stress

To date, though there are many reports ascribing an “antioxidant” role to DJ-1, there is no clear mechanism through which DJ-1 modulates cellular ROS. It was postulated that DJ-1 might act like an atypical peroxiredoxin-like molecule yet, as its capacity to “quench” ROS is so low, it suggests that there must be another mechanism underlying the neuroprotective function of DJ-1 (Andres-Mateos et al., 2007). The elucidation of a such mechanism is the subject of chapters two and three of this thesis.

1.7.1 – AKT Pathway

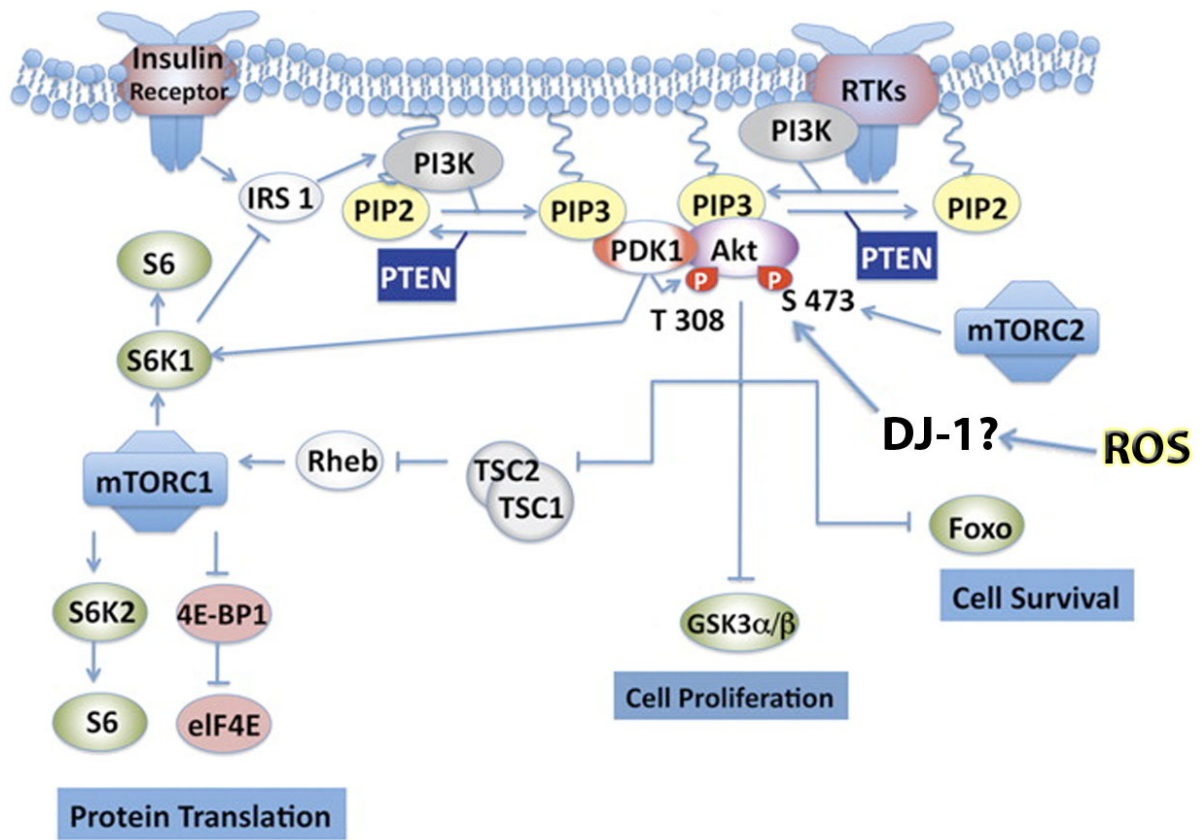
DJ-1 was first linked to the AKT pathway through a suppressor-enhancer screen where it was shown to suppress PTEN functionally in *Drosophila* (Kim et al., 2005a). This finding was particularly interesting due to the dual roles of DJ-1 in cancer and neurodegeneration. In addition, AKT has been clearly shown to play a role not only in cell

proliferation (oncogenesis) but also in neuroprotection (Hers et al., 2011; Morris et al., 2010).

1.7.1.i – AKT function

AKT (also Protein Kinase B, PKB) is a serine/threonine kinase involved in multiple cellular processes, most commonly in a pro-survival manner (Vivanco and Sawyers, 2002). It is an integral component to the PTEN/PI3K/AKT/mTOR pathway that regulates cellular proliferation, metabolism, apoptosis and transcription where it phosphorylates its downstream targets to accomplish its function (Sen et al., 2003; Vivanco and Sawyers, 2002). Upstream regulation of AKT happens at the level of PTEN and PI3K that, together, modulate transition from PIP2 to PIP3 (Figure 1.3) in an antagonizing fashion. In this way, AKT is effectively regulated via phosphatidylinositol phosphorylation and dephosphorylation. In addition, a subsequent sequence of key events must occur for AKT to become effectively activated in order to activate its downstream responsive elements. Briefly, AKT is first recruited to the plasma membrane by its Pleckstrin Homology (PH) domain that binds to PIP3. This in turn facilitates AKT phosphorylation by phosphatidylinositol dependent kinase 1 (PDK1) at threonine 308 (T308). At this point, AKT is “primed” and can be phosphorylated by the mTORC2 complex at serine 473 (S473) to become fully activated. Once AKT is phosphorylated at both T308 and S473, it is considered to be in its active state and can accomplish its kinase function within the cell. AKT phosphorylates direct downstream targets that include proteins involved in cell proliferation and survival as well as protein translation. In addition, there are multiple pathways that may be modulated by AKT (such as the NRF2 pathway mentioned in section 1.7.2) though their mechanisms remain unclear.

Figure 1.3: The PI3K-AKT Pathway in the context of oxidative stress (adapted and modified from (Iwanami et al., 2009)). A subset of AKT upstream regulators (including PDK, PTEN and PI3K) and downstream factors are presented in this diagram. Here, we postulate that upon ROS induction within the cell, DJ-1 permits activation of AKT to promote its pro-survival function.



Although much work has been done to uncover the cellular function of AKT in a cancer biology context, its relevance to neurodegeneration – specifically PD – is still poorly understood. Nevertheless, a link between AKT and PD has been suggested in models of PD including MPTP- (Shimoke and Chiba, 2001) and α -synuclein-induced toxicity (Seo et al., 2002). Indeed, trophic overexpression of AKT in animals increased dopaminergic cell size in the SNc and protected against 6-OHDA (Ries et al., 2006). Strengthening the link between AKT and PD is the observation that polymorphisms in the *AKT* gene in humans may confer protection against PD (Xiromerisiou et al., 2008). Still, the molecular mechanisms by which AKT is regulated, particularly in the context of PD-related genes, remain poorly understood in the context of degeneration.

1.7.1.ii – AKT and DJ-1

Following the first link between AKT and DJ-1 observed in *Drosophila*, a few more *in vitro* studies validated these findings and suggested that DJ-1 and AKT may indeed function in concert to protect cells from neuronal ROS (Kim et al., 2009; Vasseur et al., 2009; Yang et al., 2005). Nevertheless, these findings remained to be validated in a pre-clinical manner as well as mechanistically unveiled. Furthermore, though AKT regulates numerous of cellular pathways, better understanding of its upstream regulators and downstream effectors under specific conditions may give rise to better targets in the treatment of neurodegeneration.

1.7.2 – NRF2 Pathway

DJ-1 function in relation to the NRF2 pathway is addressed in Chapter 3. The NRF2 pathway is well established and has been the target for numerous therapies in humans. Therefore, due to its antioxidant properties, the targeting of this pathway in neurodegeneration has been logical given the high-tolerability of NRF2 activating drugs (Greco and Fiskum, 2010; van Esch, 1986).

1.7.2.i – NRF2 function

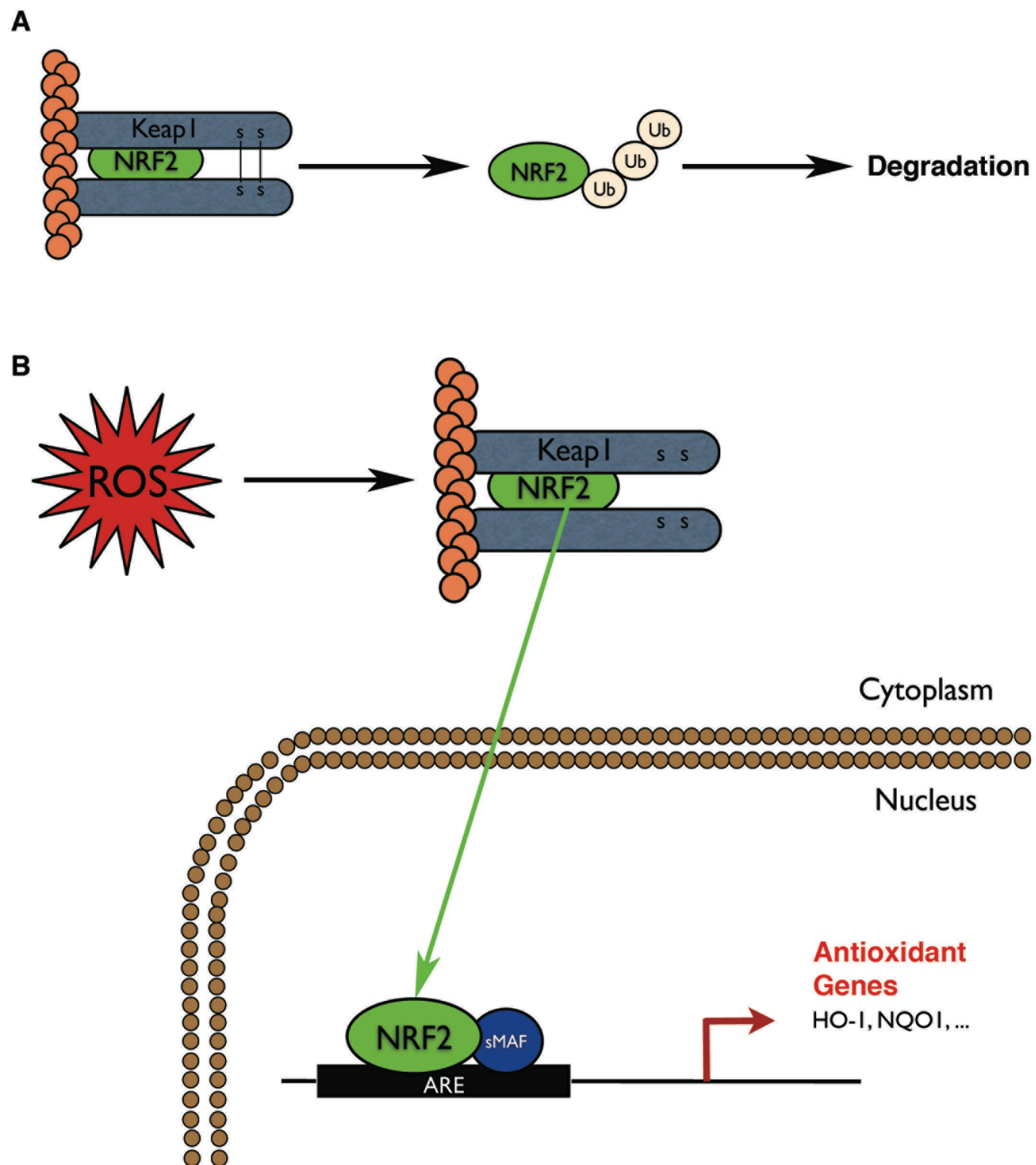
The general function of NRF2 (Nuclear Factor (Erythroid-derived 2)-Like 2; NFE2L2) is to promote cellular detoxification of xenobiotics and ROS. NRF2 is a 605 amino acid transcription factor containing an acidic activation domain in its N-terminal end and a basic leucine zipper DNA-binding domain in its C-terminal end (Moi et al., 1994). Upon its translocation to the nucleus, NRF2 promotes the transcription of a number of antioxidant genes including Heme Oxygenase 1 (HO-1), NAD(P)H dehydrogenase QuinOne 1 (NQO1) and γ -GlutamylCysteine Synthetase (γ GCS) through specific binding to an Antioxidant Response Element (ARE) in their promoter (Sykiotis and Bohmann, 2010).

1.7.2.ii – NRF2 regulation by Keap1

Under resting conditions, NRF2 is kept sequestered to the cytoplasm of cells via binding to its inhibitor KEAP1 (Kelch-like ECH-Associated Protein 1, also known as iNRF2). KEAP1, through its binding to the E3-ubiquitin ligase Cullin 3 (CUL3), allows for poly-ubiquitination of NRF2 and its subsequent degradation through the ubiquitin proteasome system. However, upon oxidative stress (or xenobiotic treatment), through mechanisms not entirely clear, NRF2 dissociates from the KEAP1-CUL3 complex and

translocates to the nucleus where it can bind the promoter of ARE-containing genes alongside its transactivating small Maf molecules (Figure 1.4). These NRF2 responsive genes in turn protect the cell from the harmful effects of ROS. In addition, the postulated mechanism by which key NRF2 activators such as *tert*-Butylhydroquinone (tBHQ) and sulforaphane work is through competitive interaction with the disulfide bonds KEAP1 that are responsible for NRF2 sequestration (Holland and Fishbein, 2010; Hong et al., 2005; Hu et al., 2011; Wakabayashi et al., 2004; Zhang and Hannink, 2003) thereby permitting NRF2 nuclear translocation.

Figure 1.4: NRF2 mechanism of action – a literature review. A) Under basal conditions, NRF2 is sequestered to an actin-bound (orange circles) KEAP1 dimer in the cytoplasm. Disulfide bonds hold this dimer together. This sequestration targets NRF2 for ubiquitination followed by degradation via the proteasome pathway. B) Under oxidative stress, NRF2 is relinquished from KEAP1 in order to translocate to the nucleus. There, it can bind to *trans-activating* small Maf proteins to bind its ARE in the promoter of key antioxidant genes (i.e. HO-1 and NQO1).



1.7.2.iii – Nrf2 and neurodegenerative disease

As NRF2 is critical for the detoxification of ROS within the cell and ROS is central to various facets of neurodegeneration, it is fairly easy to make the leap between the two. The role of NRF2 in the context of neurodegeneration has long been studied and remains a viable target for therapeutics (Calkins et al., 2009; Sykietis and Bohmann, 2010). NRF2 has been linked to stroke both clinically as well as in therapeutics. An example of this is the finding that NRF2-dependant genes are upregulated upon cellular ischemia in humans (Ghosh et al., 2011; Leonard et al., 2006). Moreover, activation of NRF2 by small molecule inducers such as *t*BHQ and Sulforaphane reduced ischemia induced brain infarct volume *in vivo* (Shih et al., 2005b; Zhao et al., 2006). In terms of PD, NRF2 has been suggested to play a role in the clinic, where it was noted to mediate the cytoprotective function of the anti-parkinsonian drug deprenyl (Nakaso et al., 2006). In addition, in the lab, the NRF2 pathway was observed to be neuroprotective against MPTP and 6-OHDA (Burton et al., 2006; Jakel et al., 2007).

1.7.2.iv – NRF2 and DJ-1

Clements and colleagues first established the link between NRF2 and DJ-1 in 2006 where, upon siRNA-mediated knockdown of DJ-1, numerous genes containing ARE motifs in their promoter were down regulated. The group further indicated that DJ-1 knockdown did not affect NRF2 mRNA but rather just its downstream effectors. Through a series of *in vitro* experiments, the group indicated that DJ-1 promoted NRF2 protein stability through inhibition of NRF2 association with its cytoplasmic inhibitor KEAP1 though they were not able to show how DJ-1 prevented this dissociation (Clements et al., 2006). Since then, however, the relationship between NRF2 and DJ-1 has been unclear due to conflicting

results. A follow up study confirmed that NRF2 expression was decreased in DJ-1 null mice subject to paraquat (Yang et al., 2007), yet another study indicated that *t*-BHQ-mediated neuroprotection was independent of DJ-1 (Gan et al., 2010). Taken together, additional clarification should be provided to effectively elucidate the biochemical and functional relationship between these two genes in the context of Parkinson's disease.

1.7.2.v – The NRF2-DJ-1 axis in relation to the AKT pathway

Previous experiments have attributed a functional link between AKT and NRF2, suggesting that the AKT pathway regulates NRF2 function (Kang et al., 2002; Martin et al., 2004; Sakamoto et al., 2009; Xiao et al., 2011). Nevertheless, the exact mechanism by which AKT regulates NRF2 is unclear as no interaction or phosphorylation of NRF2 by AKT has been presented in the literature.

1.7.3 – Other DJ-1 related pathways

DJ-1 may directly signal apoptotic pathways such as DAXX/ASK (Im et al., 2010; Junn et al., 2005; Lee et al., 2012) as well as p53 (Bretaud et al., 2007; Fan et al., 2008; Giaime et al., 2010; Shinbo et al., 2005). These are of interest though a major question remains: Why are dopaminergic cells of the SNc more susceptible than those of the VTA in patients that lack DJ-1? James Surmeier's group may have addressed this question, at least partially. In essence, DJ-1 may modulate UnCoupling Protein (UCP) expression in mitochondria, therefore loss of DJ-1 may lead to the accelerated aging of oxidant stress generating pace-maker cells of the SNc but not in those of the VTA (Guzman et al., 2010).

Though this study gives a potential explanation for the preferred sensitivity of dopaminergic cells of the SNc over those of the VTA in a PD context, it is unclear how DJ-1 modulates this phenotype. Through our work and that of others, it is possible that DJ-1

signals downstream elements that, in turn, activate pro-survival and antioxidant pathways. These findings are of particular interest but remain elusive. This signaling is a principal focus of this thesis.

In addition to acting as a signaling molecule, DJ-1 may act in a compartmentalized manner on ROS at certain subcellular locales such as the mitochondria or the plasma membrane (Canet-Aviles et al., 2004). This is an interesting concept, and is a subject of current attention in the Park lab (unpublished results, Chapter 2 of this thesis touches on this point). In addition, the transcriptional modulation effect of DJ-1 has been a target to pursue (Hod et al., 1999; van der Brug et al., 2008).

1.8 Statement of research problem, rationale and objectives:

The field of PD has received much insight from the generation of genetic animal models of the disease. A critical shortcoming in the field has been the failure of murine models to effectively recapitulate the features of the disease; particularly with regard to early-onset gene based models. As the disease may not be fully recapitulated in all its attributes, ongoing research – including work presented in Chapter 4 of this thesis – attempts to create models that better mimic the disease process. Nevertheless, the existing models previously generated by our laboratory and others have provided useful vehicles in which one could study the cellular function of these PD genes.

With regard to DJ-1, the field has been stalled in two principal aspects: 1) animal models modulating DJ-1 function (knock-outs) reveal no gross phenotypic abnormalities and 2) the cellular and molecular function of DJ-1 remains elusive. Therefore, in order to better understand how DJ-1 mutations cause early-onset PD in humans, we propose to approach the problem at the root of these two issues.

First, by continuing the backcross our DJ-1 null line from a mixed SV129/C57Bl6/J background to a more pure C57Bl6/J background, it is hoped that these mice will exhibit a more homogenous phenotype and therefore any subtle neurodegenerative that may be present can be revealed. This approach has proven to be useful to reveal better disease models not only in the field of neurodegeneration (Avdesh et al., 2011; Johnson and Wade-Martins, 2011) but also in other disease model contexts (Davie et al., 2007; Lu et al., 2010).

For one, DJ-1 has been given multiple cellular roles through which it might combat oxidative stress yet none elucidated so far appear to account for its full function. Therefore, chapters 2 and 3 of this thesis focus on elucidating a signaling mechanism through which DJ-1 may protect the cell from the deleterious effects of ROS. Interestingly, two independent recent studies have elucidated a link between DJ-1 and AKT as well as DJ-1 and NRF2. In the first study done in *Drosophila*, DJ-1 was shown to negatively regulate the tumor suppressor PTEN thereby activating the AKT pathway (Kim et al., 2005b). The AKT pathway is of interest as it has long been shown to be a key player in the cellular response to oxidative stress (Chong et al., 2005) and its function has been linked to PD (Xiromerisiou et al., 2008). Since this first study, no work has fully elucidated the role of AKT with regards to DJ-1 and animal models of PD.

The second study demonstrated that DJ-1 might facilitate NRF2 stability (Clements et al., 2006). Again, this study is of great interest as NRF2 is known as a “master antioxidant regulator” that binds to a defined ARE in the promoter of a number of phase II detoxifying genes (Venugopal and Jaiswal, 1996). In this way, NRF2 coordinates the expression of a number of antioxidant genes in response to oxidative stress.

NRF2 regulation is a complex process involving KEAP1, a cytosolic dimer that sequesters NRF2 and prevents its nuclear translocation through sulfur-bridge binding to its target to signal its degradation (Itoh et al., 1999). As the way through which NRF2 is regulated is not fully understood, particularly in a PD context, elucidating its relationship with DJ-1 is of great value.

Therefore, we hypothesize that ***DJ-1 exerts its antioxidant function through modulation of the AKT-NRF2 pathway. In addition, backcrossing of a DJ-1 null mouse to a pure C57Bl/6J background may elucidate a more clinically representative PD model.***

Overall objective:

Elucidate the molecular pathways through which DJ-1 responds to oxidative stress and establish a better murine model of PD.

Specific objectives:

Objective 1: Determine whether DJ-1 confers its neuroprotective function through AKT in a rodent model of PD.

Objective 2: Assess whether DJ-1 protects against oxidative stress-mediated damage through its downstream effector NRF2.

Objective 3: Determine whether backcrossing DJ-1 null mice to a pure C57Bl/6J background may result in a clinically relevant rodent model of Parkinson's disease.

The three objectives stated above are addressed in Chapters 2, 3 and 4, respectively.

Chapter 2: DJ-1 Protects the Nigrostriatal Axis from the Neurotoxin MPTP by Modulation of the AKT Pathway.

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* = Authors contributed equally

Statement of author contribution

This manuscript described the functional interaction between DJ-1 and the PI3K/AKT pathway in preventing neuronal death. We show that, in our experimental paradigm of neuronal death induced by oxidative stress, AKT is upregulated and that this regulation is dependent on DJ-1. This AKT-mediated neuroprotection through DJ-1 is involved in both *in vitro* (H₂O₂) and *in vivo* (MPTP) models of cell death. Finally, we suggest a mechanism through which DJ-1 may activate AKT through its recruitment to membranous fractions of the cell. Together, this body of data suggests a role of AKT mediated neuroprotection in a rodent model of PD that is dependent on the PD gene DJ-1.

Maxime Rousseaux and Hossein Aleyasin (co-first authors) designed the experiments. Hossein Aleyasin predominantly performed *in vitro* experiments shown in figure 4.1 (panels A and B), Figures 4.2 and 4.3 with assistance from **Maxime Rousseaux**. **Maxime Rousseaux** predominantly performed the *in vivo* experiments presented in figure 4.1 (panels C and D) and the entire figure 4.4 with assistance from Hossein Aleyasin. The first co-authors have been assisted by Paul C. Marcogliese, Sarah J. Hewitt, Alvin P. Joselin, Dr. Isabella Irrcher, and Mohammad Parsanejad, and supervised and guided by Dr. David S. Park. Dr. Raymond H. Kim from the University of Toronto has generated the original DJ-1 null mice and contributed reagents and analytical tools. Patricia Rizzu contributed in this work by providing technical assistance and culturing human derived lymphoblast cells. Steve M. Callaghan produced adenovirus vectors, used for both *in vitro* and *in vivo* experiments. Maxime Rousseaux, and Paul C. Marcogliese, performed histological preparations and assessments. Sarah J. Hewitt aided with membrane fractionations and cell culture. **Maxime**

Rousseaux, Hossein Aleyasin, and Dr. David Park carried out data analysis. **Maxime Rousseaux**, Hossein Aleyasin, and Dr. David Park redacted the paper, with collaboration and scientific input from Dr. Ruth Slack and Dr. Tak W. Mak.

***DJ-1 Protects the Nigrostriatal Axis from the Neurotoxin MPTP by
Modulation of the AKT Pathway.***

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Number of text pages: 24

Number of figures: 5

Abstract

Loss of function DJ-1 (PARK7) mutations have been linked with a familial form of early onset Parkinson's disease (PD). Numerous studies have supported the role of DJ-1 in neuronal survival and function. Our initial studies utilizing 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 H₂O₂ *in vitro* and *in vivo* following 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, we 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.

Introduction

Individuals with homozygous loss-of-function mutations of DJ-1 (PARK7) have been clinically characterized with familial early onset Parkinson's disease (PD) (Abou-Sleiman et al., 2003; Bonifati et al., 2003). Although the physiological role of DJ-1 is not been completely understood, several lines of evidence indicate a protective role for DJ-1 in multiple models of neuronal and non-neuronal oxidative stress-induced cell death (Canet-Aviles et al., 2004; Gu et al., 2009; Inden et al., 2006; Lev et al., 2009; Taira et al., 2004). For instance, 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 (Kim et al., 2005b). 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 (Lavara-Culebras and Paricio, 2007; Martinat et al., 2004; Yang et al., 2005). 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 (Aleyasin et al., 2007).

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

(Andres-Mateos et al., 2007; Taira et al., 2004). 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 (Andres-Mateos et al., 2007; Yang et al., 2005). Several alternative mechanisms to account for the neuroprotective function of DJ-1 have been suggested. For instance, via its putative role in transcription regulation (Xu et al., 2005), DJ-1 upregulates the expression of other antioxidant genes such as glutathione synthase (GS) during oxidative stress (Zhou and Freed, 2005). Interestingly, it has also been reported that DJ-1 enhances the activity of the transcription factor Nrf2, a master regulator of antioxidant genes (Clements et al., 2006; Malhotra et al., 2008). Alternatively, DJ-1 has also been shown to modulate key signaling pathways (Gu et al., 2009; Yang et al., 2005). One signaling pathway implicated with DJ-1 function and relevant to the present work is AKT (van der Brug et al., 2008; Yang et al., 2005).

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 [C]). AKT has an N-terminus pleckstrin homology (PH) domain that mediates the interaction of AKT with a plasma membrane phospholipid, phosphatidyl inositol 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 (Klippel et al., 1997; Kohn et al., 1996). Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is particularly known for its action to convert phosphatidylinositol-3, 4, 5- triphosphate (PIP3) to phosphatidylinositol-4, 5- biphosphate (PIP2). This function of PTEN directly antagonizes phosphatidylinositol-3- kinases (PI3K) to eventually down regulate AKT (Stambolic et al., 1998; Sun et al., 1999). Several lines of evidence indicated that the AKT signaling pathway responds to oxidative

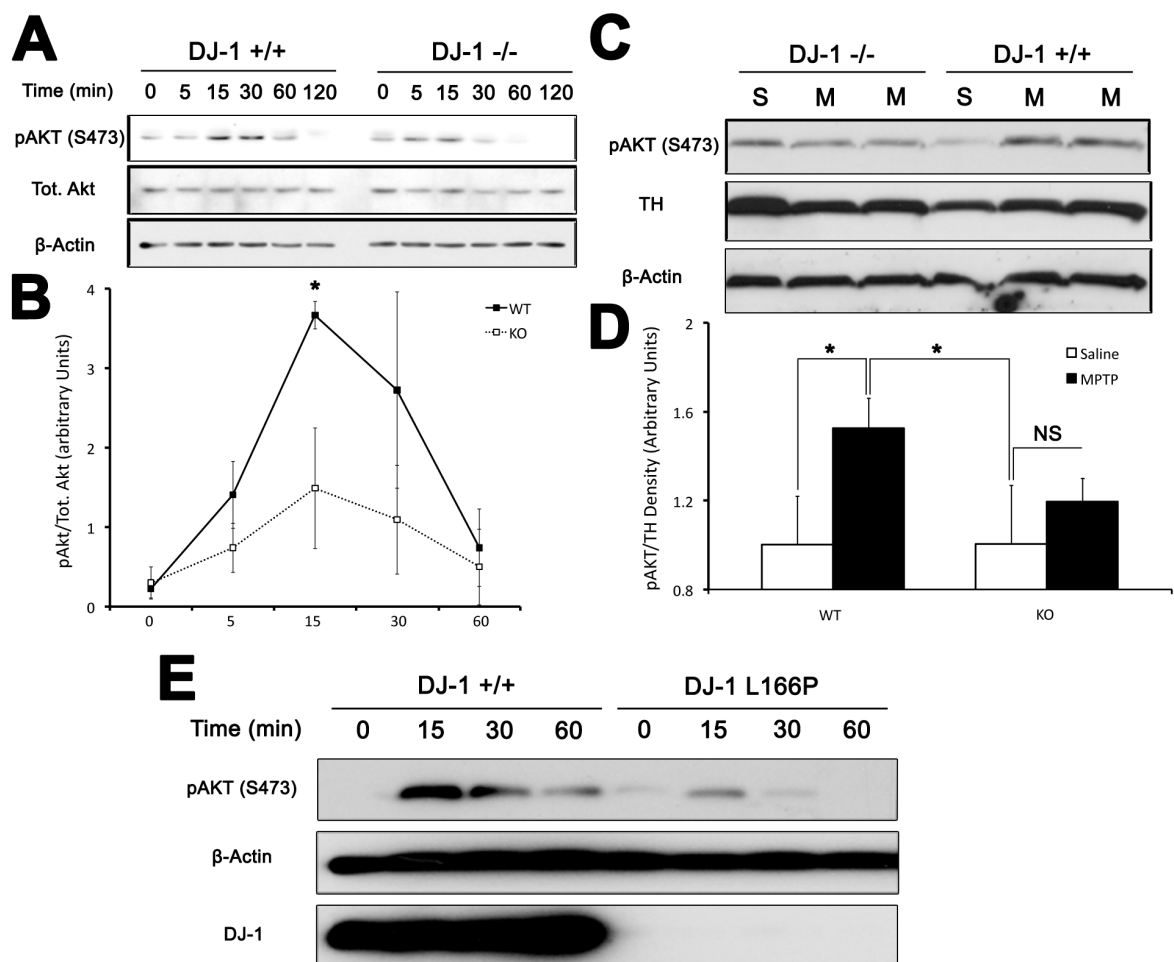
stress (Crossthwaite et al., 2002) and exerts a neuroprotective function (Lee et al., 2009; Sun et al., 2009). Moreover, a large number of studies *in vitro* have illustrated that pharmacological compounds which protect cells against oxidative stress exert their neuroprotective effects through activation of the AKT pathway (Dudek et al., 1997; Heo et al., 2009; Li et al., 2008b; Liu et al., 2009; Malagelada et al., 2008).

Early studies described DJ-1 as a negative regulator of PTEN using a drosophila genetic screen (Kim et al., 2005a). Evidence to confirm this negative regulation was demonstrated via down regulation of DJ-1 using small interfering RNA, which resulted the inhibition of endogenous AKT phosphorylation in cancer cell lines as well as in the drosophila brain (Kim et al., 2005a; Sitaram et al., 2009; Yang et al., 2005). Furthermore, loss of DJ-1 has been shown to reduce AKT activation in response to hypoxia in murine embryonic fibroblasts (MEFs, (Vasseur et al., 2009)). 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 addition, based on our results, we propose a mechanism suggesting that DJ-1 acts as an upstream regulator of AKT through membrane recruitment in order 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 mM H_2O_2 for indicated time points. As demonstrated in Fig. 2.1A, phosphorylation of AKT peaked in wild type neurons at 15 minutes whereas in the knockout, there was a reduction in AKT phosphorylation. Quantification of three independent experiments revealed a significant reduction in p-AKT 15 minutes following treatment (3.67 ± 0.17 in DJ-1^{+/+} vs. 1.49 ± 0.76 in DJ-1^{-/-}) as demonstrated in Fig. 2.1B. 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. 2.1C, and quantified in Fig. 2.1D, 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. 1E, AKT response was significantly attenuated in L166P mutated cells compared to the controls.

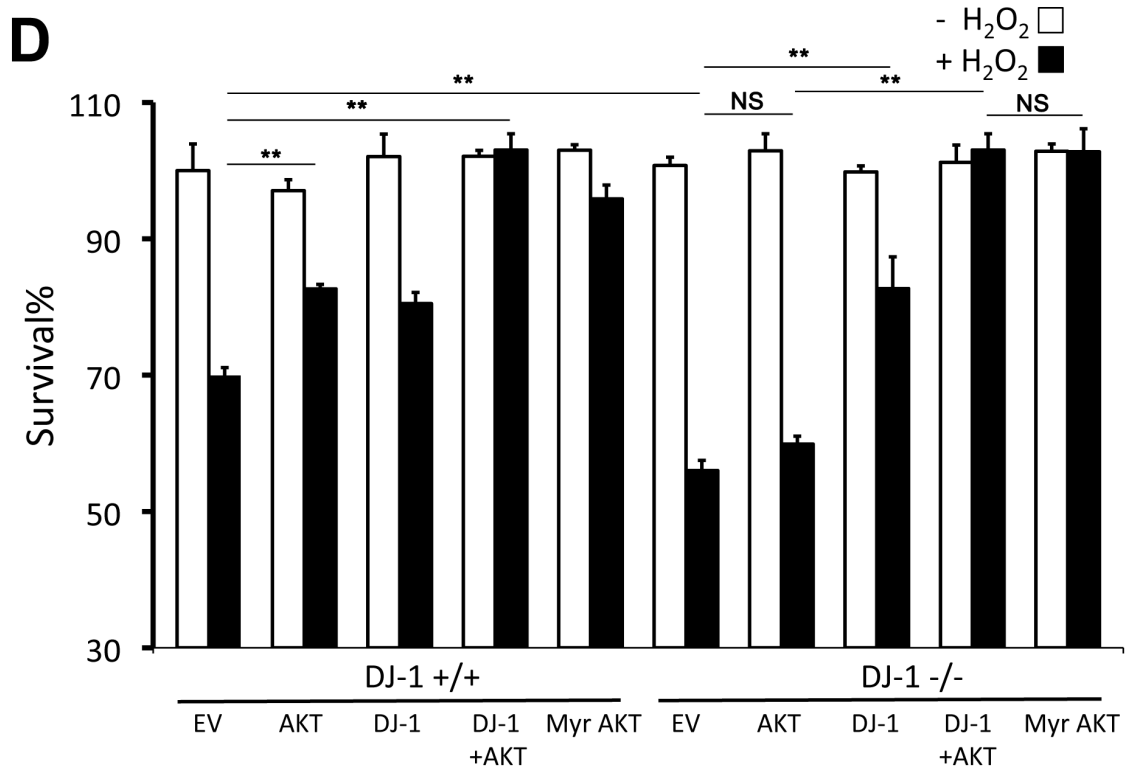
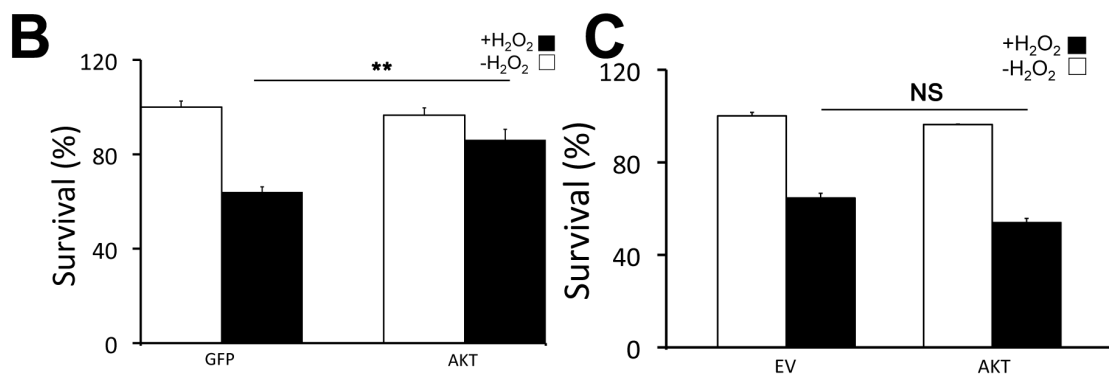
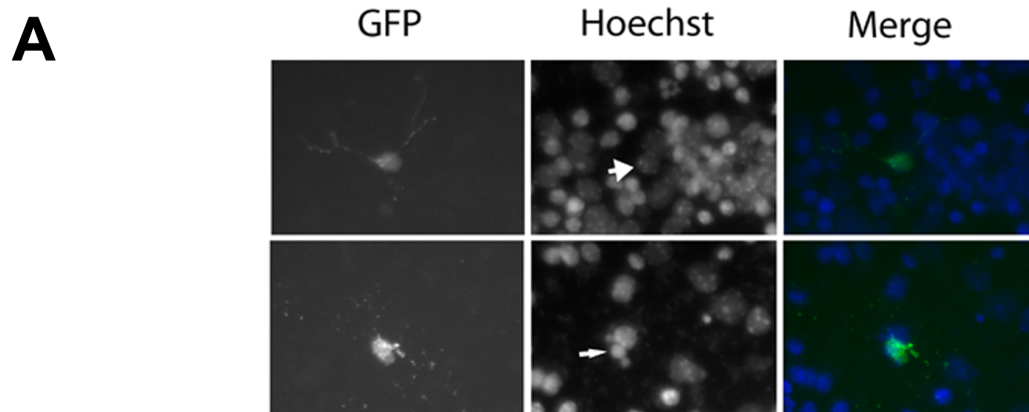
Figure 2.1: AKT activation is suppressed in the absence of DJ-1. (A) Cortical neurons from DJ-1 $+/+$ and DJ-1 $-/-$ embryos were harvested, plated and treated with H_2O_2 ($100\mu M$) in a time-dependant fashion. Extracts were probed for pAKT (S473), Total AKT and β -actin by western blot. (B) Quantification of (A) from 3 independent experiments. Values are presented as mean optical density relative to total AKT. (C) 8-10 week old C57Bl6 mice of WT and DJ-1 KO genotype were treated with two 30mg/kg doses of MPTP (M), or saline (S), given 24h apart. 3h 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-course. Analysis of cell lysates was carried out by Western blot. Picture presented is representative of 2 independent experiments. Data is presented as mean $\pm$ S.E.M.



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 H₂O₂ *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 H₂O₂, 24 hours after transfection, and survival was assessed as described in Methods (Fig. 2.2A). As shown in Fig. 2.2B, induction of exogenous wildtype AKT confers protection in DJ-1^{+/+} neuronal cells in response to H₂O₂. Next, DJ-1^{-/-} cortical neurons were tested to examine whether induction of wildtype AKT could provide similar protection in DJ-1 deficient cells. Surprisingly, induction of exogenous AKT failed to protect DJ-1^{-/-} neurons against H₂O₂-induced death (Fig. 2.2C). 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 wildtype AKT together with or without a DJ-1 expression vector, DJ-1 only, or myristoylated AKT, a membrane-anchored constitutively active form of AKT (Meier et al., 1997). After treatment with H₂O₂, cell survival was assessed. The results of this experiment clearly verified our findings in Fig. 2.2C, 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. 2.2D). Interestingly, myristoylated AKT significantly protects neurons against oxidative damage induced by H₂O₂ regardless of DJ-1 genotype (95.85±2.02 % DJ-1^{+/+} vs. 102.77±3.38 % DJ-1^{-/-}).

Figure 2.2: AKT requires DJ-1 to exert its neuroprotective function *in vitro*. (A)

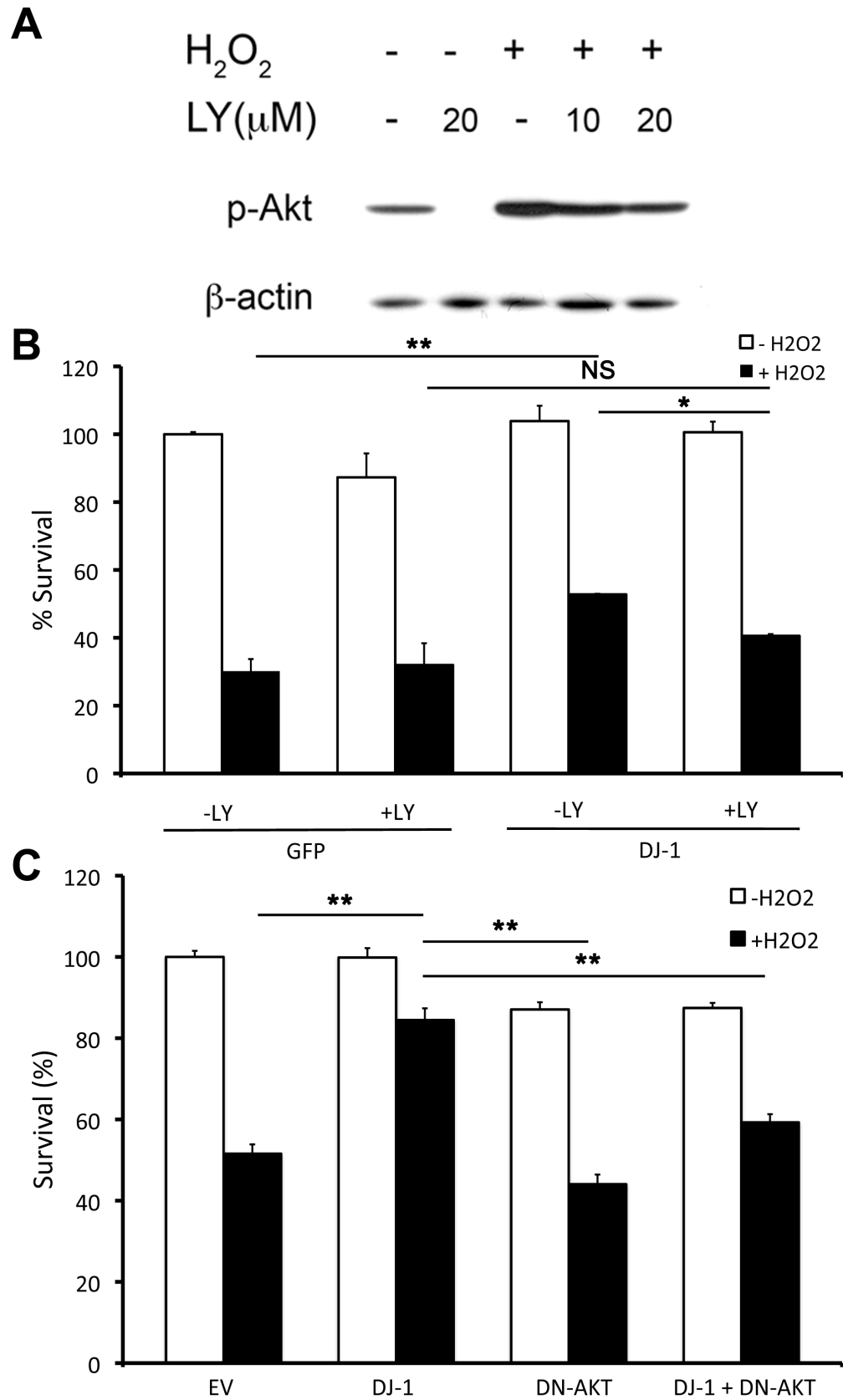
Representative pictures of alive (*top panel*, large arrowhead) and dead (*bottom panel*, thin arrowhead) neurons. Neuronal survival was measured by identifying GFP-positive cells and determining their nuclear integrity by Hoechst stain. (B) Cortical neurons from either DJ-1^{+/+} or ^{-/-} embryos were harvested, plated and transfected with empty vector (EV), AKT, DJ-1 or Myr-AKT. Cells were then treated with H₂O₂ (30μM) or vehicle control (-H₂O₂) for 3h. Quantification was assessed as in (A). Data is presented as mean ± S.E.M.



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. In order to examine this, we first utilized a conventional pharmacological inhibitor of AKT, LY294002 (LY; (Taylor et al., 2004). Since 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. 2.3A). 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 10mM LY or vehicle for 30 minutes before application of H₂O₂ or vehicle for 3 hours. Cells were then assessed for survival. As shown in Fig. 2.3B, 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 utilized 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 towards endogenous AKT (Stambolic et al., 1998). As shown in Fig. 2.3C, 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).

Figure 2.3: DJ-1 requires AKT activation to promote cellular survival *in vitro*. (A)

Cortical neurons were treated for either 10 or 20 μ M of LY with and without H₂O₂ (100 μ M, 15 minutes) to determine the effective dose of LY. (B) Cortical neurons were infected with either GFP or DJ-1 with GFP. Cells were then pre-treated with LY followed by H₂O₂ treatment for survival assessment. (C) Cortical neurons were co-transfected with GFP and empty vector (EV), DJ-1, DN-AKT or a DJ-1/DN-AKT combination. Survival was assessed as in (B). Data is presented as mean $\pm$ S.E.M.



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 H₂O₂. To confirm these results and to test this hypothesis in a more clinically relevant paradigm, we examined whether induction of wildtype AKT can protect nigrostriatal neurons against the dopaminergic specific neurotoxin MPTP *in vivo*. To achieve this, we injected adenoviral vectors harboring HA tagged wildtype AKT, or myristoylated AKT into the striatum of DJ-1^{+/+} and DJ-1^{-/-} age-matched mice. β -gal expressing adenoviruses were used as control. As shown in Figure 2.4B and 2.4C, 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 Figure 2.4A. 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 MTN (Fig. 2.4E). 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 $\pm$ 8.5 vs. 109.9 $\pm$ 9.2, respectively). Meanwhile, there was no significant difference between ipsi- and contralateral sides of the SNc in the KO animals that received HA-AKT. (88.2 $\pm$ 7.4 vs. 90.9 $\pm$ 8.0, respectively). To verify whether the loss of TH immunoreactivity was in fact due to 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 $\pm$ 1.9% vs. 50.7 $\pm$ 3.0%, ipsilateral vs. contralateral) whereas no protective effect was observed when injecting HA-AKT in the DJ-1^{-/-} mice (48.9 $\pm$ 3.0% vs.

51.9±4.0% ipsilateral vs. contralateral) (Figure 2.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. 2.4F). 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.

Figure 2.4 AKT requires DJ-1 to exert its neuroprotective function in an *in vivo* model of Parkinson's disease. (A) Schematic representation of treatment course. Mice were injected ipsilaterally in the striatum with adenovirus (LacZ, HA-AKT, Myr-AKT) 7 days prior to 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 labelling of both TH and protein of interest in the SNc. (C) Representative pictures of both Striatum and SNc of mice treated with MPTP or Saline. SNc and Striatum were stained for TH and DAT, respectively. (D) 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. (E) Quantification of DAT positive fibres normalized to cortex (DAT-negative). Data is presented as mean $\pm$ S.E.M.

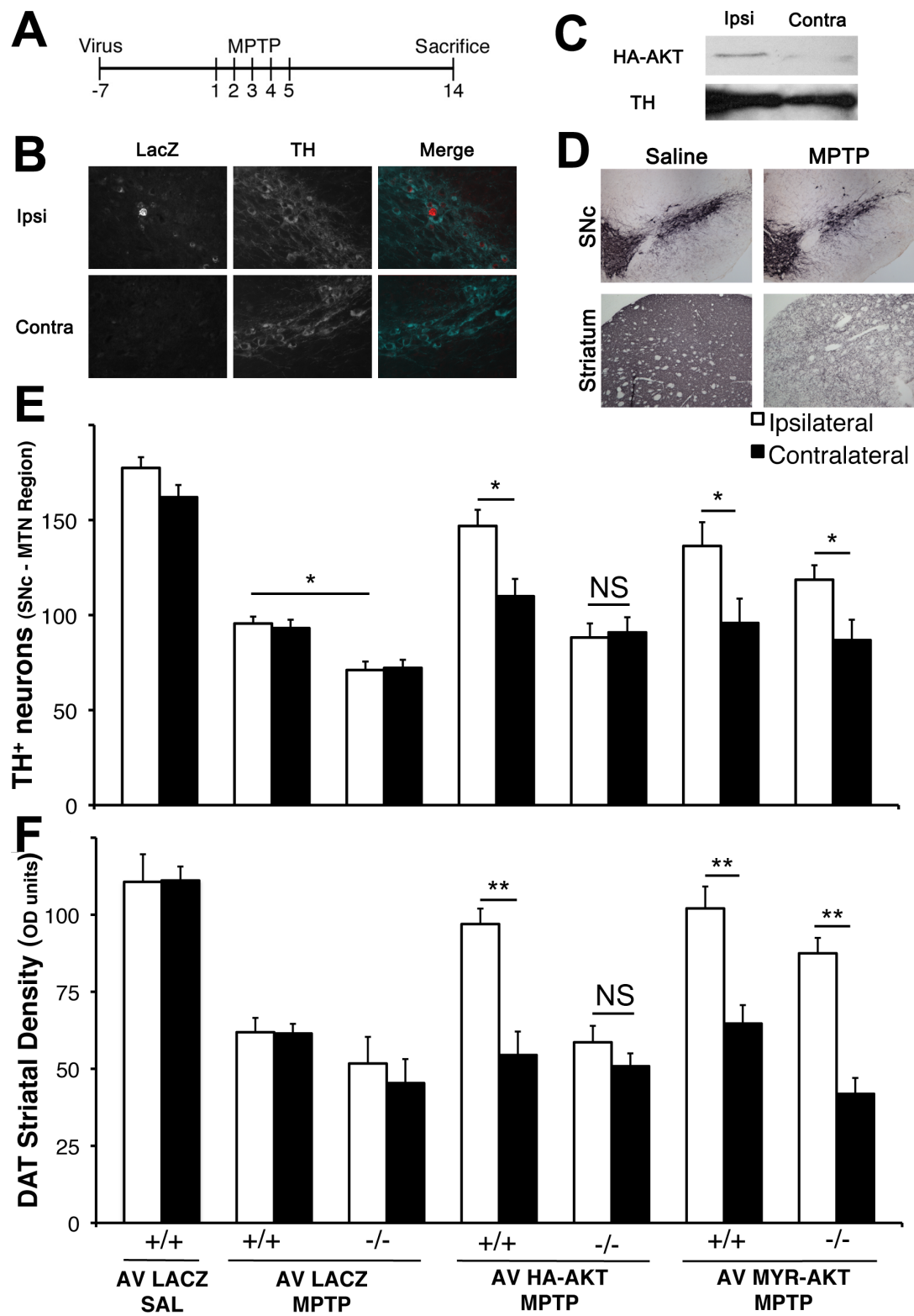
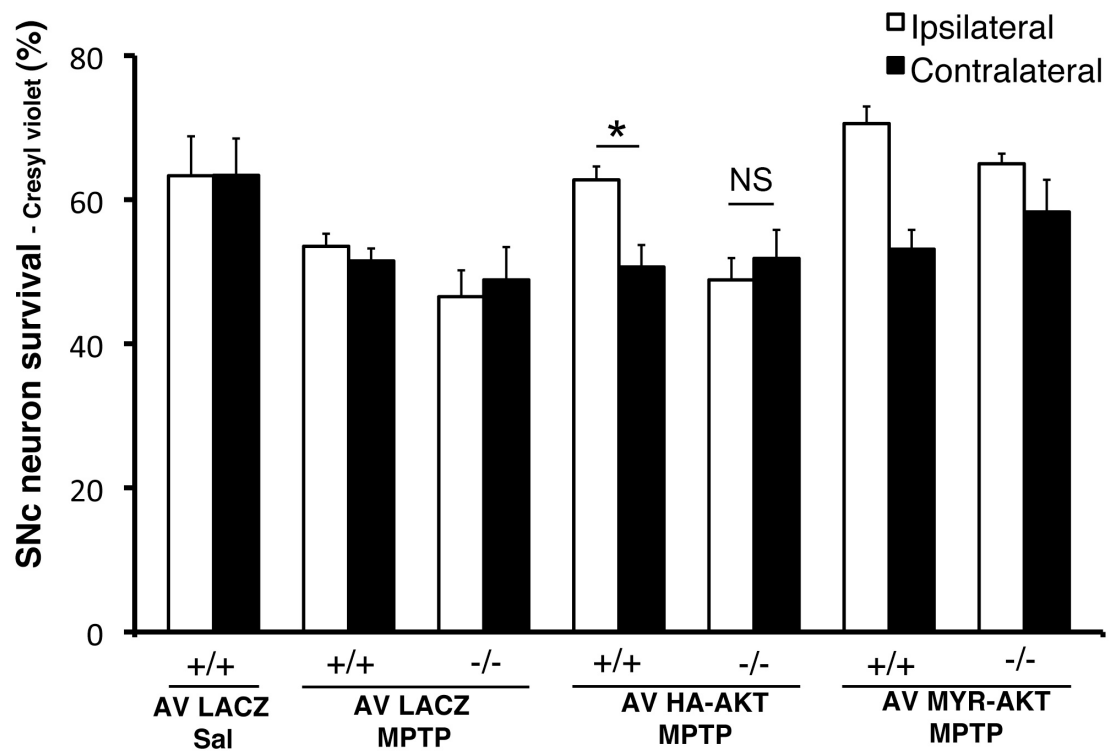


Figure 2.S1: AKT requires DJ-1 to exert its neuroprotective function in an *in vivo*

model of Parkinson's disease: Cresyl Violet evidence. Cresyl violet staining was performed at the level of the MTN region of the SNc where virus expression was highest. Round nuclei with visible nucleoli were considered alive and shrunken, fragmented nuclei were quantified as dead. Survival was assessed as the ratio of alive over total number of cresyl violet stained cells. "Ipsi" denotes the side of the brain ipsilateral to the virus injection whereas "contra" denotes the contralateral side.

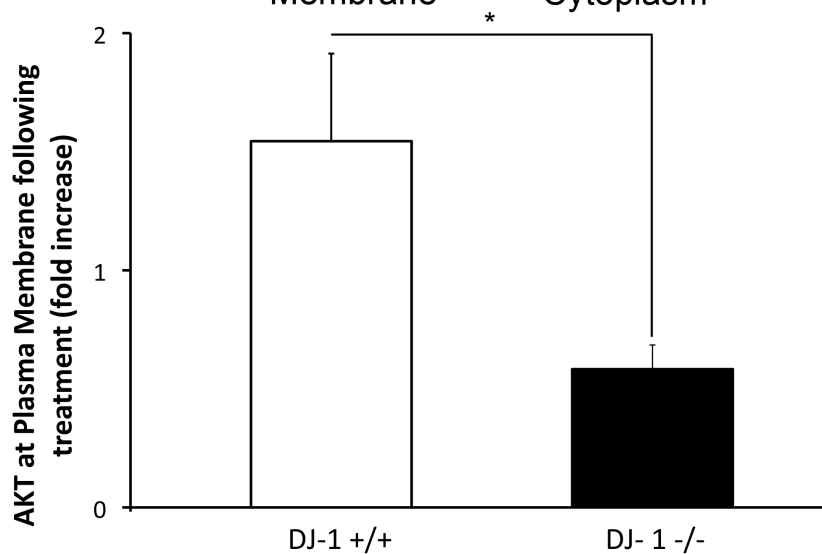
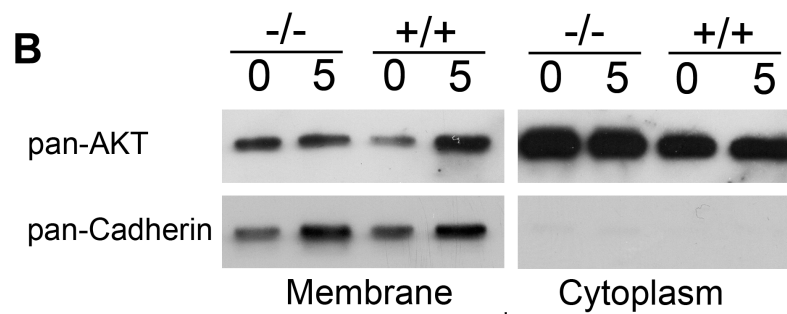
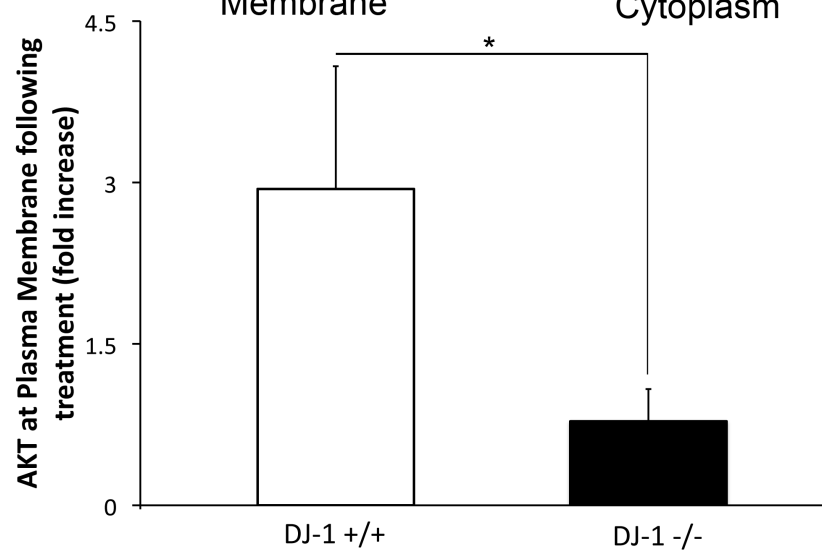
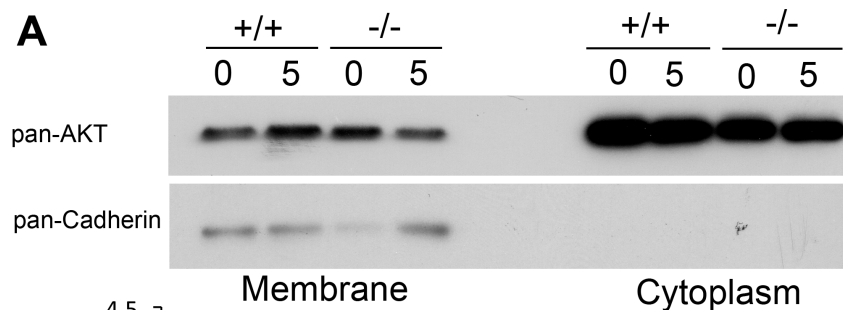


DJ-1 modulates AKT translocation to membranous fractions. Our results *in vitro* and *in vivo* demonstrated specifically that myristoylated rather than a wild type form of AKT promotes protection in DJ-1^{-/-} neurons. We therefore tested whether DJ-1 was affecting AKT translocation to membranous compartments following oxidative stress. This was done by determining the subcellular localization of AKT following H₂O₂ treatment in DJ-1^{+/+} and DJ-1^{-/-} neurons and MEFs. As shown in Fig 2.5A, the H₂O₂-induced AKT localization to the membrane fraction is greatly decreased in the DJ-1^{-/-} compared to 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 H₂O₂ (0.58±0.10 fold increase) whereas DJ-1^{+/+} neurons showed an AKT translocation 5 minutes post-treatment (1.55±0.37 fold increase) No significant differences were observed in levels of total AKT in the cytoplasmic fraction.

Figure 2.5 AKT requires DJ-1 to localize to membranous fractions following insult. A)

DJ-1^{+/+} and ^{-/-} MEFs were treated with 500 μ M of H₂O₂ for 5 minutes or with media control. Western blot analysis by probing pan-AKT as well as 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 is representative of n=4 experiments.

B) DJ-1^{+/+} and ^{-/-} cortical neurons were subjected to 100 μ M of H₂O₂ for 5 minutes or media control. Quantification was performed as in A. Data is presented as mean $\pm$ S.E.M.



Discussion

DJ-1 was first discovered as a weak oncogene with an unclear mechanism of action (Nagakubo et al., 1997). 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, PIAS α (Takahashi et al., 2001) as well as RNA-protein interactions (Hod et al., 1999). The DJ-1 protein also displayed some homology to the proteins of the ThiJ/PfpI family of bacterial proteases (Mitsumoto et al., 2001; Wilson et al., 2004) 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 which link this protein to neurodegeneration, PD, and oxidative stress. These themes include, but are not limited to the following: 1) DJ-1 protects neurons against oxidative stress (Inden et al., 2006; Lavara-Culebras and Paricio, 2007; Liu et al., 2008a; Martinat et al., 2004; Meulener et al., 2005a; Paterna et al., 2007; Yokota et al., 2003), 2) 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 (Goldberg et al., 2005; Park et al., 2005; Pisani et al., 2006; Yamaguchi and Shen, 2007), and 3) DJ-1 modulates signaling pathways critical to cell survival such as PTEN and AKT, at least in select non-neuronal contexts (Kim et al., 2005a).

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, while 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* (Salinas et al., 2001) as well as 6-OHDA treatment *in vivo* (Ries et al., 2006). Since AKT recruitment to the membrane is a prior event to its phosphorylation and activation (Franke et al., 1997; James et al., 1996), these results, in addition to the cell fractionation experiments presented in our study, suggested that DJ-1 permits AKT translocation to the membrane fractions.

Taken together, our study 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 of 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 H₂O₂ can be altered depending on antioxidant protein activity within the cell (Endo et al., 2007; Handy et al., 2009; Taylor et al., 2005). Thus, further studies in models which 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 in order to permit AKT activation, although this needs to be further investigated in more physiologically relevant models (Kim et al., 2009). 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 play a role (Alessi et al., 1997; Sarbassov et al., 2005; Stambolic et al., 1998). Thus, additional studies should be performed to investigate the nature of the DJ-1/AKT interdependence.

Finally, it is interesting to note that while 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 (Xiromerisiou et al., 2008). This observation provides further strength to the notion that the DJ-1/AKT signaling axis may be important in regulating dopaminergic

function/death. Elucidation of these mechanisms may provide an eventual basis for neuroprotective therapies.

Materials and Methods

Cell culture, Western blot analysis as well as *in vivo* stereotaxic injections and **MPTP administration** were performed as previously described (Qu et al., 2007). For additional *in vivo* and *in vitro* procedures refer to *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 2000rpm for 3min. The cell pellet was resuspended in 200uL of hypo-osmolar buffer (50mM Tris-HCl, pH 7.4; 50mM NaCl; protease inhibitor complex (Roche)) and homogenized for 30 sec. Samples were centrifuged at 14,000rpm, at 4°C for 20min. Supernatants (cellular debris) were then transferred to 1.5mL ultracentrifuge tubes (Beckman) and centrifuged at 50,000rpm, at 4°C for 3hr. The pellets (microsomal enriched) were resuspended in RIPA buffer (150mM NaCl; 1% NP-40; 0.5% deoxycholic acid; 0.1% SDS; 50mM 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 $\pm$ SEM. Significance at $p < 0.05$ (*), $p < 0.01$ (**) and NS denotes no significant difference.

Acknowledgements

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Supplemental Information (SI)

Materials and methods

Cell culture: Embryonic cortical neurons were harvested from wild type or DJ-1 null littermate embryos as described (Xiang et al., 1996). For survival experiments, at 4 days *in vitro* (DIV), co-transfection of target plasmids (Gene of interest + GFP as a marker of transfected cells) was performed using Lipofectamine 2000 transfection reagent (Invitrogen). The following day, cells were treated with 30 μ M H₂O₂ for 3h. Neurons were fixed with 4% PFA/Picric acid in PBS and survival was assessed by evaluating the nuclear integrity of GFP-positive cells. Alternatively, viral vectors were introduced at time of plating (Multiplicity of infection = 60) and left to express for 36h before being subjected to treatment. In biochemical experiments, at 2 DIV, cells were treated with 100 μ M H₂O₂ for various times and cells were harvested for Western blot analysis. Murine Embryonic Fibroblasts (MEFs) were harvested at the same time as cortical neurons as previously described (Camarasa et al., 2009). Cell transformation and immortalization was achieved by transducing a SV40 construct as adapted from Smith *et al* (Smith et al., 1970). Antibodies used for analysis were pAKT (Ser 473, CST), total AKT (CST), β -actin (Sigma), pan-Cadherin (Abcam), DJ-1 (SCBT), HA (CST) and β -galactosidase (Promega).

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 2000rpm for 3min. The cell pellet was resuspended in 200 μ L of hypo-osmolar buffer (50mM Tris-HCl, pH 7.4; 50mM NaCl; protease inhibitor complex (Roche)) and homogenized for 30 sec. Samples were centrifuged at 14,000rpm, at 4°C for 20min. Supernatants (cellular debris) were then transferred to 1.5mL ultracentrifuge tubes

(Beckman) and centrifuged at 50,000rpm, at 4°C for 3hr. The pellets (microsomal enriched) were resuspended in RIPA buffer (150mM NaCl; 1% NP-40; 0.5% deoxycholic acid; 0.1% SDS; 50mM Tris-HCl pH 8.0) and sonicated briefly for subsequent Western blot analysis. Supernatants from final spin were used as a cytoplasmic control.

***In vivo* adenoviral gene delivery and MPTP administration:** As described previously, recombinant adenoviruses that are injected into the striatum retrogradely translocate and express on the ipsilateral SNc at the level of the Medial Terminal Nucleus (MTN) (Crocker et al., 2003). Brain samples from all experimental groups were harvested and analyzed by Western blot and immunohistochemistry in order to confirm virus expression. Samples were probed for markers of the virus of interest. Ipsilateral sides were compared to their contralateral counterparts. 7 days post-virus injection, mice underwent a sub-chronic MPTP regimen. For biochemistry, 3 hours following the second MPTP injection, brains were quickly removed and SNc were dissected and flash-frozen.

Immunohistochemistry and assessment of neuronal loss *in vivo*: After being perfused transcardially, mice brains were fixed and cryoprotected as described (Crocker et al., 2001). Midbrain sections containing the SNc and striatal sections were immunostained as previously described (Mount et al., 2007). At least 3 sections per brain were chosen at the level of the MTN and TH positive neurons were counted on sides both ipsilateral and contralateral to the virus injection. Striatal DAT quantification was performed at 200X. For each picture, 20 samples of striatum and 3 samples of cortex were used for analysis. Relative intensity was measured using NIH ImageJ. Three pictures per animal were used to calculate the average striatal density.

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 $\pm$ SEM. Significance at $p < 0.05$ (*), $p < 0.01$ (**) and NS denotes no significant difference.

Chapter 3: In Vivo Neuroprotection by DJ-1 is Governed by its Regulation of the Master Antioxidant Regulator NRF2

Manuscript in preparation

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Aleyasin, Steve M. Callaghan, Tak W. Mak, Timothy H. Murphy, Jenny P.Y. Ting, Ruth S.
Slack, David S. Park

Statement of author contribution:

This manuscript describes a link between the neuroprotective function of DJ-1 and NRF2, a master antioxidant regulator. We demonstrate both *in vitro* and *in vivo* that the hypersensitization observed in DJ-1 null mice following oxidative stress can be effectively rescued by viral reintroduction of NRF2. We further show that NRF2 acts downstream of DJ-1 as hypersensitivity to ROS seen in NRF2-null neurons cannot be rescued by DJ-1 overexpression *in vitro*. We also note that NRF2-mediated neuroprotection relies on AKT function. Furthermore, we demonstrate that DJ-1 may mediate some of the antioxidant properties of NRF2 through transient binding to its endogenous cytosolic inhibitor KEAP1 *in vitro*. Taken together, this data suggests that NRF2 is a novel factor mediating the function of the DJ-1/AKT neuroprotective axis.

Maxime Rousseaux designed the experiments. **Maxime Rousseaux** performed the majority of the experiments with assistance from Katie Don-Karolis, Paul C. Marcogliese and Dianbo Qu. Katie Don-Karolis performed immunoprecipitation experiments with DJ-1 and KEAP1. The project was supervised and guided by Dr. David S. Park. Dr. Tak W. Mak's laboratory from the University of Toronto has generated the original DJ-1 null mice and contributed reagents and analytical tools. Steve M. Callaghan generated the adenoviral constructs. Hossein Aleyasin provided scientific and technical input. Timothy H. Murphy and Jenny P.Y. Ting provided NRF2 null mice and analytical tools. **Maxime Rousseaux** and Dr. David Park carried out data analysis. **Maxime Rousseaux** and Dr. David Park redacted the manuscript, with collaboration and scientific input from Dr. Ruth Slack and Dr. Tak W. Mak.

***In Vivo Neuroprotection by DJ-1 is Governed by its Regulation of
the Master Antioxidant Regulator NRF2***

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Abstract:

DJ-1, a gene linked to familial PD, encodes a protein with putative roles in transcriptional regulation, post-translational SUMOylation and cell transformation. Although the exact mechanisms underlying PD are unknown, common molecular pathways involving reactive oxygen species (ROS) likely exist. To this end, recent evidence suggests that DJ-1 acts as a regulator of the oxidative stress response. A key factor in the cell that mediates the detoxification of ROS through the transcriptional regulation of numerous antioxidant genes is NRF2. DJ-1 is reported to stabilize NRF2 upon its induction by *t*BHQ, which leads to its nuclear compartmentalization and subsequent transcriptional activation. However, the exact mechanism by which DJ-1 affects NRF2 under oxidative stress conditions and whether this is critical in neuronal death is unknown. Presently, we show that NRF2 expression can protect neurons from MPP⁺/MPTP treatment *in vitro* and *in vivo*. Protection in this case is observed in both the presence and absence of DJ-1. In contrast, however, overexpression of DJ-1 fails to protect neurons exposed to mitochondrial stress in the absence of NRF2 suggesting that DJ-1 acts upstream of NRF2. In addition, we note that NRF2 may mediate its neuroprotective effects through the pro-survival function of AKT. This finding is congruent with the notion that DJ-1 mediates its neuroprotective function through AKT. Importantly, we also show that DJ-1 forms a transient – oxidative stress dependent – complex with the NRF2 cytosolic inhibitor KEAP1. We propose that DJ-1 confers its antioxidant function through NRF2 according to the oxidative status of the cell. This may be done in a direct (through inhibition of KEAP1) or indirect (through activation of AKT and subsequent activation of NRF2) manner. Taken together, this data suggests the importance of a DJ-1 – Nrf2 link in oxidative stress-induced neuronal injury.

Introduction:

Parkinson's disease (PD) is the most common movement neurodegenerative disorder affecting ~1-2% of the population over 60 (Farrer, 2006). It is pathologically characterized by both the loss of dopaminergic neurons in the *Substantia Nigra pars compacta* (SNc) as well as the presence of intracytoplasmic inclusions termed Lewy Bodies (LBs). Though the etiology of this progressive degenerative disorder remains unclear, a growing body of evidence suggests that reactive oxygen species (ROS) and the cellular damage they elicit may be at the root of the disease process. For instance, ROS and its end products have been shown to be increased in *post-mortem* PD patient brains (Schapira et al., 1989; Schapira et al., 1990). In addition, mitochondrial complex I inhibitors that generate high levels of ROS have been shown to elicit Parkinsonism in mice and humans alike (Betarbet et al., 2000; Hallman et al., 1984; Kaul et al., 2003; Langston et al., 1983; Li et al., 2003). Moreover, genes linked to PD – particularly autosomal recessive PD – have been likened to have important function in the detoxification of ROS within the cell; be it by permitting proper mitochondrial homeostasis of ROS or directly quenching the free radicals (Cookson, 2010; Schapira, 2008). Therefore, targeting of ROS in a PD context has been a sought after therapeutic avenue as it appears to be a common feature of the degenerative process

Though primarily sporadic, the emergence of genes linked to PD has provided great insight into the pathogenesis of the disease. Of these genes, DJ-1 (*PARK7*) was identified in 2003 as a causative factor for autosomal recessive early-onset PD (Bonifati et al., 2003). Since then, manipulations of DJ-1 in various biochemical and physiological contexts have assigned multiple functions to the protein. One in particular is its capacity as a signaling

molecule to combat oxidative stress initiated by reactive oxygen species (ROS) (Andres-Mateos et al., 2007; Junn et al., 2009; Shendelman et al., 2004; Zhong and Xu, 2008).

Numerous studies have endeavored to elucidate the mechanism through which DJ-1 combats oxidative stress. For instance, some studies have suggested that DJ-1 may act on ROS in a direct, scavenging manner (Andres-Mateos et al., 2007), while others have suggested that DJ-1 may alleviate the damaging effects of ROS by affecting the translation of pro-survival and antioxidant genes (van der Brug et al., 2008). Finally, emerging studies have indicated a putative signaling role for DJ-1 whereby it may coordinate the detoxification of ROS through downstream regulation of cell survival/death molecules (McNally et al., 2011; Mo et al., 2010). For instance, in a previous study, we noted that DJ-1 could confer its neuroprotective role in the nigro-striatal axis through the pro-survival gene AKT (Aleyasin et al., 2010). In addition, another study suggested that DJ-1 might confer its neuroprotective function by stabilization of the master antioxidant regulator Nuclear Factor Erythroid 2 like-2, NRF2 (Clements et al., 2006).

NRF2 confers its antioxidant and neuroprotective function through transcriptional regulation of a number of detoxifying genes. Under basal conditions however, NRF2 is sequestered to the cytoplasm by its inhibitor KEAP1 (Kelch-like ECH Associated Protein 1 or iNRF2). KEAP1 is bound to the actin cytoskeleton and to Cullin3, an E3-ligase that facilitates the poly-ubiquitination and subsequent degradation of NRF2 by the proteasome. Thus, classic NRF2 activators such as *t*BHQ (*tert*-butylhydroquinone) and Sulforaphane act by preventing the KEAP1-NRF2 association and thus facilitate the nuclear translocation of NRF2. In the study by Clements and colleagues, NRF2 activation via *t*BHQ in cancer cell lines was impaired in the absence of DJ-1. The authors suggested that this might be done

through a mechanism by which DJ-1 prevents the association between NRF2 and KEAP1, though no clear interaction was observed between this putative complex machinery. In addition, *t*BHQ itself is known to act through the *direct* regulation of KEAP1. Therefore, the mechanism underlying DJ-1-mediated NRF2 stabilization in this model is likely not done through interaction with KEAP1 but rather by a different mechanism. Moreover, another study in neurons clearly indicated that in neurons, *t*BHQ could protect regardless of the DJ-1 genotype indicating that DJ-1 was not critical in mediating *t*BHQ-mediated NRF2 induction. Collectively, this would suggest that cancer lines might act differently, particularly in relation to neurons when it comes to *t*BHQ-mediated KEAP1 induction. Taken together, in this study, we sought to determine more carefully: a) whether NRF2 is critical in models of PD and b) whether this is modulated by factors potentially regulated by DJ-1, namely AKT and KEAP1.

Though these studies and others have hinted towards the pro-survival signaling effect of DJ-1, full elucidation of a mechanism through which it mediates neuroprotection in a degenerative context is crucial in understanding the pathogenesis of PD and establishing future therapeutic avenues. To this end, the objective of this study is to examine the DJ-1 – NRF2 relationship both on a biochemical and physiological level in pre-clinical models of PD.

Results:

To bridge the gap between DJ-1 and NRF2 in a functional manner, we devised rescue experiments using *in vitro* and *in vivo* models of PD. We have previously observed that DJ-1^{-/-} neurons are hypersensitive to oxidative stress (Kim et al., 2005b). Nevertheless, the mechanism through which loss of DJ-1 sensitizes these neurons remains unclear. Therefore, to test whether NRF2 may act in a similar pathway to DJ-1, we examined whether NRF2 overexpression could mitigate the hypersensitivity to oxidative stress in DJ-1^{-/-} neurons. To do so, we infected cultured DJ-1^{+/+} and DJ-1^{-/-} cortical neurons with NRF2 or control (GFP) adenoviral vectors and subjected these transduced cells to MPP⁺ insult (Figure 3.1). Survival assessment revealed that NRF2-mediated protection could occur in the DJ-1^{+/+} neurons. This finding is consistent with previous reports indicating a pro-survival role for NRF2 in a culture setting (Lee et al., 2003; Shih et al., 2005a). Furthermore, viral delivery of NRF2 could provide efficient protection against the hypersensitivity to MPP⁺ observed in DJ-1^{-/-} neurons. Thus, NRF2 overexpression appears to bypass the loss of DJ-1 and can protect neurons regardless of their DJ-1 genotype in this cellular PD model.

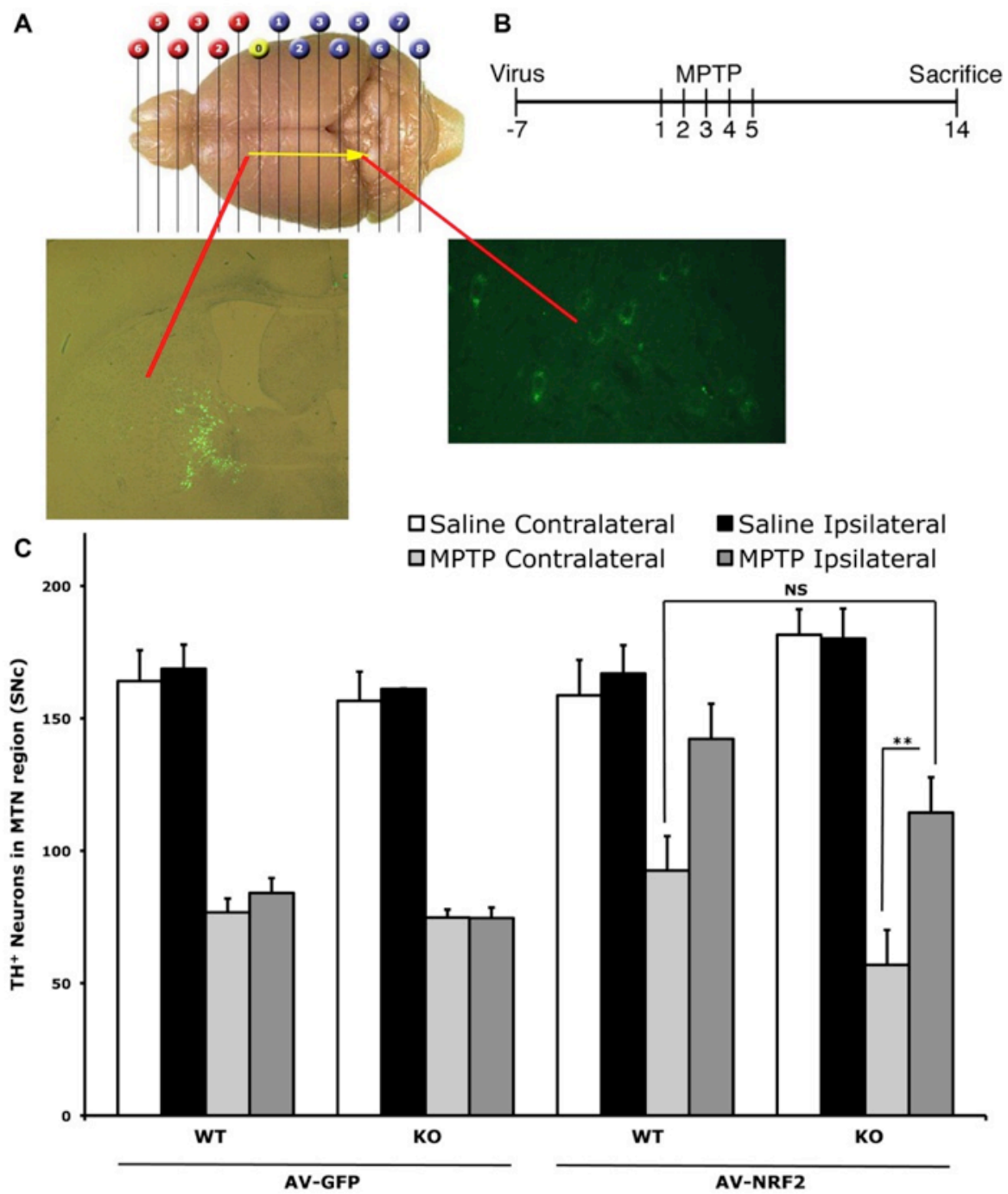
Figure 3.1. Overexpression of NRF2 can rescue the hypersensitisation seen in DJ-1

null neurons *in vitro*. (A) Cortical neurons from DJ-1 null or wild-type embryos overexpressing adenoviruses carrying NRF2 or GFP control were treated with 50 μ M MPP⁺ and assessed for survival via nuclear morphology staining colocalizing with GFP positive cells. Thin arrows depict shrunken, picnotic, nuclei while thick arrow depict live nuclei via Hoechst staining. (B) White bars depict media treated cells whereas black bars depict MPP⁺ treated cells, n=4 DJ-1^{+/+}, n=5 DJ-1^{-/-}.

To validate whether this *in vitro* finding could translate into a pre-clinical model of PD, we tested whether viral overexpression of NRF2 could rescue the hypersensitivity observed in DJ-1 null dopaminergic neurons following administration of the dopaminergic neurotoxin MPTP. Indeed, similarly to our *in vitro* findings, we noted a significant neuroprotection at the level of the SNc in both the DJ-1^{+/+} and DJ-1^{-/-} mice that received NRF2 following MPTP treatment, whereas no protection was offered in GFP treated mice or on any of the contralateral sides of the injection (Figure 3.2).

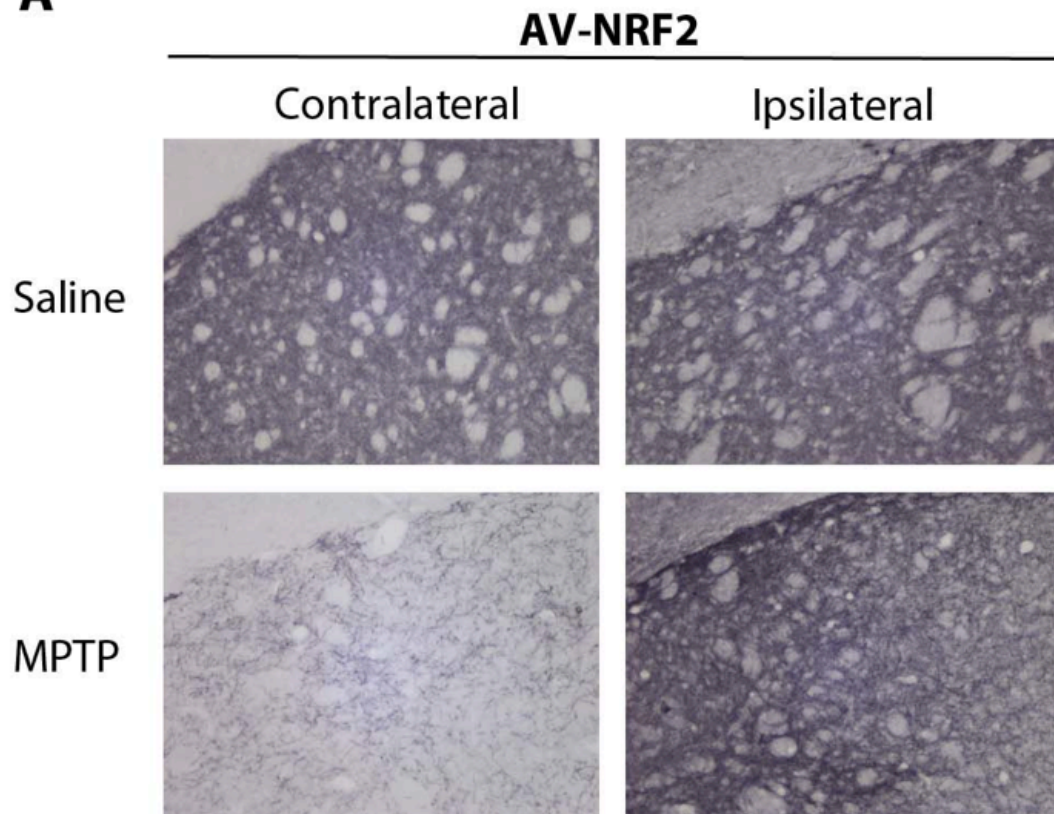
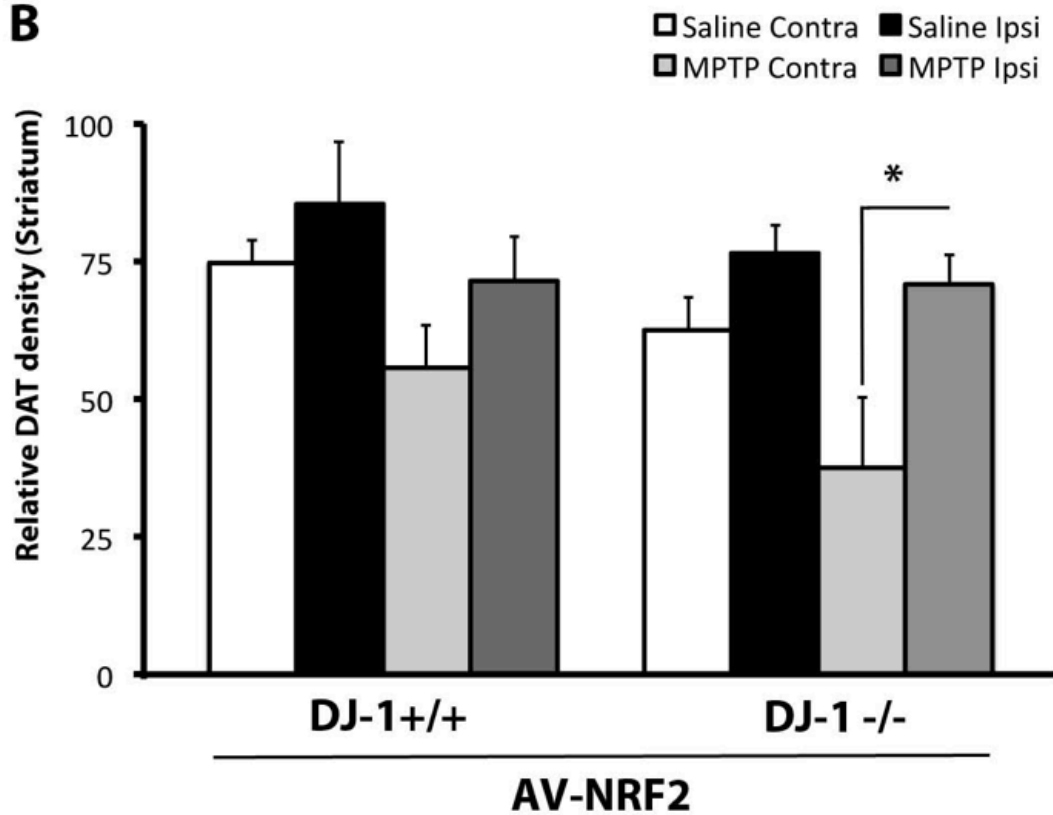
Figure 3.2. Overexpression of NRF2 via adenoviral mediated gene delivery can

rescue the hypersensitisation to MPTP in the SNc of DJ-1^{-/-} mice. (A) Mice were injected unilaterally with adenoviruses expressing either GFP or NRF2 containing GFP on a separate promoter. Expression was assessed to verify retrograde transport from the striatum to the SNc 7 days post injection. 7 days following injection, mice received sub-chronic MPTP paradigm and were sacrificed 14 days later (schematized in B). (C) Survival was assessed by quantification of TH-immunoreactive neurons 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. Numbers are representative of n=3-6 animals per group. All data are presented as mean $\pm$ SEM. Significance at $P < 0.01$ (**) and NS denotes no significant difference.



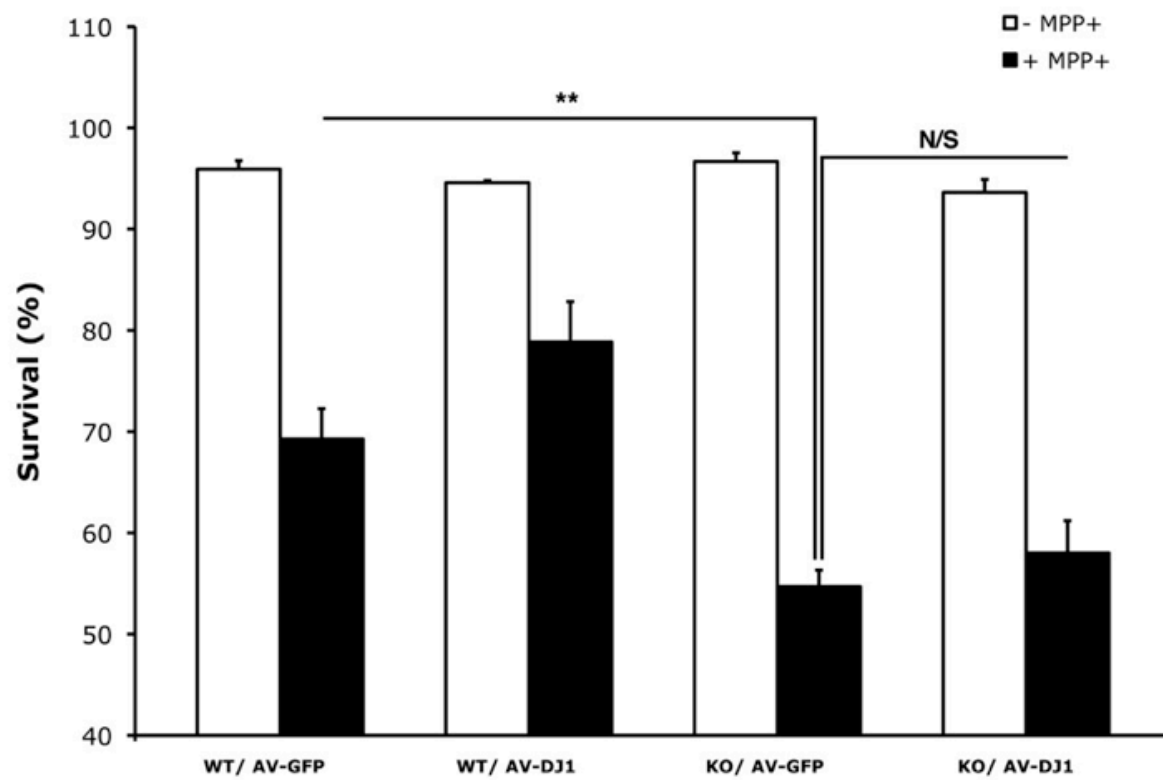
Moreover, to test to see whether NRF2-mediated neuroprotection occurred not only at the soma of the dopaminergic cells but also at the functional location of their terminals, striatal sections were examined for DAT immunoreactivity. Indeed, similar to the SNc, unilateral neuroprotection was observed in both DJ-1^{+/+} and DJ-1^{-/-} mice overexpressing NRF2 at the level of the striatum (Figure 3.3).

Figure 3.3. Overexpression of NRF2 via adenoviral mediated gene delivery can rescue striatal denervation in DJ-1^{-/-} mice subjected to MPTP. A) Representative sections from the striatum of DJ-1^{-/-} mice stained for DAT. Sections are representative of virus-injected side (ipsilateral) and control side (contralateral to injection) as well as with MPTP or Saline treatment. Densitometry of relative DAT expression was performed and quantified for each group in (B). Samples are representative of $n = 3-5$ animals per group. All data are presented as mean $\pm$ SEM. Significance at $P < 0.05$ (*)

A**B**

Taken together, the results thus far suggest that NRF2, likely through its pro-antioxidant function, can protect the nigro-striatal axis from degeneration and can even do so in the absence of DJ-1. To ascertain whether DJ-1 mediates its neuroprotective effects through NRF2 however, we performed the converse experiment. In this case, we obtained primary cortical neurons from NRF2^{+/+} and NRF2^{-/-} embryos, overexpressed DJ-1 or control (GFP) adenoviruses and subjected the cells to MPP⁺ insult (Figure 3.4). As previously reported, DJ-1 overexpression could provide protection in NRF2^{+/+} cells from MPP⁺ (Aleyasin et al., 2010; Kim et al., 2005b). In addition, NRF2^{-/-} cortical neurons were hypersensitized to MPP⁺, a finding also supporting previous results (Chen et al., 2009). Interestingly, however, DJ-1 overexpression in the NRF2^{-/-} neurons could not provide effective prophylaxis against neurotoxic injury. Collectively, these findings would suggest that DJ-1 acts upstream of NRF2 *through* NRF2 to perform its neuroprotective role.

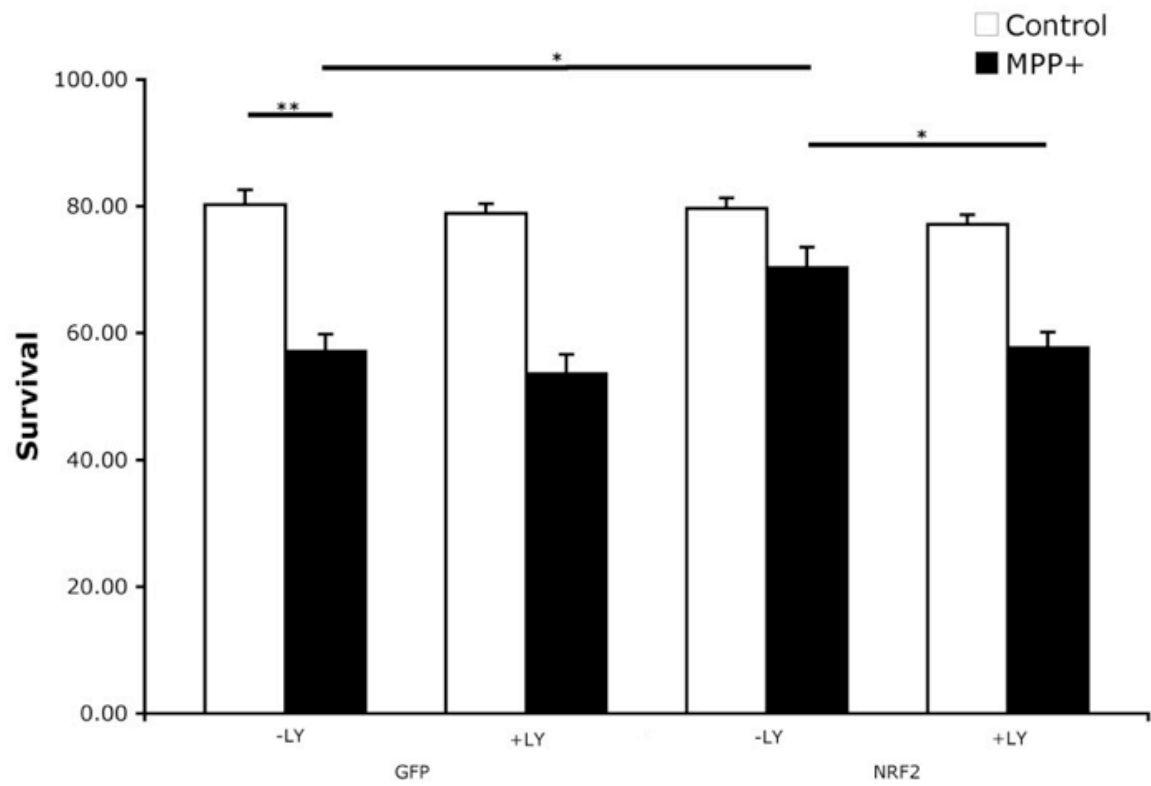
Figure 3.4. NRF2 acts downstream of DJ-1 *in vitro*. (A) Cortical neurons from NRF2^{-/-} or NRF2^{+/+} embryos were transduced with adenoviruses overexpressing DJ-1 or GFP at time of plating. At 2 days *in vitro* (DIV), cells were treated with 50 μ M MPP⁺ for 24h, fixed and assessed for survival via nuclear morphology staining (Hoechst) colocalizing with GFP positive cells. $n = 4$, triplicates. All data are presented as mean $\pm$ SEM. Significance at $P < 0.01$ (**) and NS denotes no significant difference.



We next aimed to identify a mechanism through which the DJ-1-NRF2 axis is mediating its neuroprotective effects. In this regard, we previously identified AKT as a key factor in DJ-1-mediated neuroprotection. Under neurotoxic insult, without proper AKT function, DJ-1-mediated protection was hampered (Aleyasin et al., 2010). As we and others have shown, NRF2 expression is neuroprotective (Shih et al., 2005a; Shih et al., 2005b). Interestingly, previous studies have indicated that AKT may increase NRF2 function (Kang et al., 2002; Sakamoto et al., 2009). Therefore, we sought to establish whether the DJ-1-AKT neuroprotective pathway might mediate its activity through NRF2. To do so, we first tested whether AKT promoted the neuroprotective effects of NRF2 in a cellular model of PD. In our *in vitro* model, we noted that adenoviral-mediated delivery of NRF2 protects cortical cultures from the neurotoxin MPP⁺. Interestingly, pre-treatment of the culture with the PI3K/AKT pathway inhibitor LY294002 abolishes this NRF2-driven protection (Figure 3.5). This finding, in conjunction with our previous observations that DJ-1 loss dampens AKT activation, suggests that the DJ-1-AKT pathway regulates NRF2 protection function. How this occurs however remains unknown. Verification of NRF2 expression and subcellular localization in this model was not possible due to a failure in antibody specificity. Nevertheless, it is likely that pre-treatment of LY294002 in this system abolishes NRF2-induced gene expression following MPP⁺ as has been previously suggested (Rojo et al., 2008; Wang et al., 2012). This hypothesis is currently being explored.

Figure 3.5. The PI3K-AKT pathway is essential for NRF2 mediated protection

following MPP⁺ in primary cortical neurons. Neurons from CD-1 wild-type cultures were transduced with NRF2- or GFP-carrying adenoviruses and pre-treated with the PI3K inhibitor LY294002 (LY) before being treated with MPP⁺ (20 μ M) for 48h. Survival was assessed by identifying live/dead nuclear staining colocalizing with GFP positive neurons. White bars represent vehicle treated cells whereas black bars represent MPP⁺ treated groups. All data are presented as mean $\pm$ SEM. Significance at $P < 0.05$ (*), $P < 0.01$ (**).



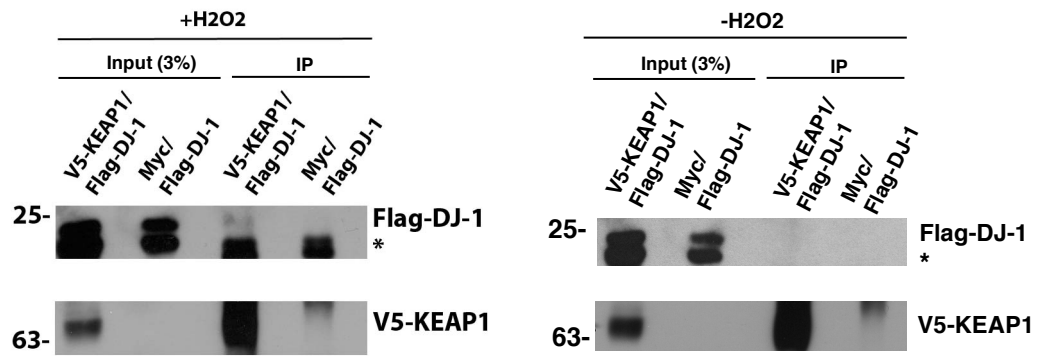
Our previous results suggest that DJ-1 requires AKT to mediate NRF2 function. This may be due to a number of factors such as regulation of the transcriptional activity of NRF2 (Sakamoto et al., 2009), or the nuclear translocation of NRF2 via phosphorylation and inhibition of GSK3 β (Salazar et al., 2006). These notions are under current investigation. However, another possibility is that DJ-1 may *directly* regulate NRF2 stability. In this regard, we hypothesized that it may form a complex with the cytosolic NRF2 sequestering protein KEAP1. To address this, we performed co-immunoprecipitation of DJ-1 and KEAP1. Importantly, this was done in the presence and absence of a brief oxidant stimulus by hydrogen peroxide (H₂O₂; 5 minutes 300 μ M) as well as in reducing (+DTT) and non-reducing (-DTT) conditions. Upon pull-down of V5-KEAP1, we could detect DJ-1 presence only when cells were pre-treated with H₂O₂ (+H₂O₂) and not when in their native state (-H₂O₂). Moreover, this observation was only made in the non-reducing conditions whereas in the reducing conditions, no interaction was observed (data not shown). Pull-down of myc (control vector) alone did not reveal any DJ-1 in its complex. Accordingly, we observed a complex formation between DJ-1 and KEAP1 that appeared transient and dependent on the presence of H₂O₂ and an oxidative cellular environment (Figure 3.6).

Figure 3.6. DJ-1 may transiently interact with KEAP1 following oxidative stress.

HEK-293 cells were co-transfected with V5-KEAP1, Flag-DJ-1, or control vectors.

Cells were treated with H₂O₂ (300 μ M) or vehicle control (-H₂O₂) for 5 minutes.

Extracts were incubated with anti-V5 antibody at 4°C overnight, followed by incubation with anti-mouse Ig IP beads at 4°C for 1 hour. Protein complexes were subjected to SDS-PAGE and detected by Flag and V5 by Western blot. * denotes a non-specific band.



Discussion:

In this present study, we set out to determine whether DJ-1 may mediate its neuroprotective effects through the master antioxidant regulator NRF2. To do so, we tested whether DJ-1 positively acts on NRF2 to allow for cellular detoxification following neuronal insult. First; we noted that expression alone of NRF2 was protective in our models of PD. Second; we tested whether this protection required DJ-1. Hypersensitivity initially observed in DJ-1 null mice to oxidative stress can be rescued by the prophylactic overexpression of NRF2 both *in vitro* and *in vivo*. Interestingly, at least *in vitro*, the converse experiment revealed that DJ-1 was not capable of rescuing hypersensitivity of NRF2 null neurons from oxidative stress. This finding is particularly interesting as it suggests that at least a portion of the neuroprotective function of DJ-1 is mediated through its downstream effector NRF2. Third; we asked how NRF2 protective function might be regulated by functionally exploring two cellular pathways: The PI3K-AKT pathway and the KEAP1-Cul3 pathway. In the first case, we hypothesized that if DJ-1 indeed served its neuroprotective role through NRF2, it may do so using AKT as an intermediate element by modulating the transcriptional activity and/or nuclear localization of NRF2. In our model of degeneration, at least *in vitro*, the neuroprotective effects of NRF2 are mediated through the PI3K pathway. Moreover, these functional findings are supported by the notion that DJ-1 may mediate the neuroprotective effects of NRF2 in a biochemical fashion. A recent study published by Im *et al.*, indicated a role for DJ-1 in regulating TRX1 expression through NRF2 following oxidative insult *in vitro* (Im et al., 2012). These findings parallel ours in that the group reported decreased TRX1 expression in DJ-1 null neurons following ROS and indicated that this phenomenon occurs in parallel of AKT activation and that it is also dependent on NRF2 binding to an

ARE in the promoter of TRX1. They further indicated that this event was independent of DJ-1 binding to KEAP1 under basal conditions.

Another possibility accounting for this DJ-1-mediated NRF2 response is that DJ-1 may respond to oxidative insult in a more direct manner by regulating pathways that directly affect NRF2 stability such as KEAP1. Indeed, our study indicates that the interaction between DJ-1 and KEAP1 is transient in nature and likely depends on the oxidative status of the cell. Lastly, a putative joint mechanism through which the complex formation between DJ-1 and KEAP1 may be mediated through AKT is also plausible. For instance, DJ-1 might permit KEAP1 or NRF2 phosphorylation by AKT – a finding suggested to occur at least in *C. Elegans* (Tullet et al., 2008) – and subsequently permit the dissociation of this complex and the translocation of NRF2 to the nucleus.

The collective findings in neurons versus those in cancer cell lines with regard to this pathway are different in that DJ-1 activity appears to be independent of *t*BHQ in neurons. In support of this notion are the findings by Gan and colleagues indicating that *t*BHQ can protect neurons in the presence and absence of DJ-1 (Gan et al., 2010). In their model, *t*BHQ likely acts independently from a DJ-1-AKT-NRF2 axis and directly inhibits KEAP1 through its previously established mechanism (Satoh et al., 2006). This finding is congruent with the theory that DJ-1 acts as an *endogenous* ROS-sensing molecule that may bind KEAP1 in a similar mechanistic way to *t*BHQ following oxidative insult.

Taken together, DJ-1 might act as a central ROS sensor to regulate multiple aspects of NRF2 regulation including post-translational modifications, subcellular localization and stability. The specific domain of DJ-1 responsible for its neuroprotective function remains elusive. Nevertheless, much insight has been provided as to the role of its cysteine 106

residue with regards to its antioxidant and neuroprotective function. For instance, Canet-Aviles and colleagues demonstrated that DJ-1 could translocate to mitochondria upon oxidation of its cysteine 106 residue to a sulfinic acid (Canet-Aviles et al., 2004). This sulfinic acid-driven mitochondrial localization was inhibited upon introduction of a C106A substitution mutant. Moreover, studies from our laboratory and others have confirmed the role of this cysteine residue in models of neurodegeneration including cerebral ischemia and PD (Aleyasin et al., 2007; Miyazaki et al., 2008). Taken together, if the C106 residue of DJ-1 indeed plays a critical role in mitigating ROS-dependent cellular damage, perhaps it does so through disulfide binding to KEAP1 to relinquish NRF2 to the nucleus. Indeed, in our study complex formation between DJ-1 and KEAP1 following oxidative insult could be abolished in reducing conditions (+DTT) thereby suggesting that this interaction may occur through a disulfide bond. This again supports the notion that DJ-1 may act like an endogenous version of the small molecule NRF2 activators (via KEAP1 inhibition), *t*BHQ and sulforaphane as the latter two are thought to act on KEAP1 through competitive disulfide bridge binding thereby disrupting the KEAP1 homodimer enclosure wherein NRF2 lies (Sato et al., 2006; Wakabayashi et al., 2004; Zhang et al., 2004)). Lastly, as mentioned above, the regulation of NRF2 by DJ-1 may be done independent of KEAP1 and rather directly or indirectly through AKT. These putative pathways are summarized in Figure 3.7.

Figure 3.7. Model: The DJ-1-AKT-NRF2 axis of neuroprotection. A) Under resting conditions, NRF2 is sequestered to the cytoplasm by KEAP1. KEAP1 is in turn conjugated to the actin cytoskeleton and to the E3-ubiquitin ligase adaptor Cul3. Sequestered NRF2 can then be polyubiquitinated by Cul3 and degraded by the proteasome (lighter shade = degraded). AKT under steady state exists in the cytosol and remains relatively unphosphorylated. B) Following generation of ROS, DJ-1 is oxidized and permits AKT translocation to the plasma membrane where it can be phosphorylated and can subsequently modulate downstream elements (including NRF2) to protect the cell. In addition, DJ-1 can disrupt the KEAP1-NRF2 interaction and permit subsequent NRF2 translocation to the nucleus where it can activate a slew of antioxidant genes to detoxify the cell from ROS.

Future interaction studies examining the role of DJ-1 mutants (Particularly C106A, a mutant incapable of forming disulfide bonds) in the DJ-1/KEAP1 complex formation will likely shed more light onto the nature of this interaction. Moreover, *in vitro* biochemical studies to study the relationship between AKT and KEAP1/NRF2 will likely delineate the nature of this pathway in the context of DJ-1-mediated neuroprotection. If DJ-1 indeed protects the cell from toxic insult in one of these manners, potential therapies for PD may lie in activators that impact the C106 residue of the protein such as those described by Miyazaki and colleagues (Miyazaki et al., 2008).

Experimental Procedures:

Animal generation and maintenance:

DJ-1^{-/-} and NRF2^{-/-} mice were generated as previously described (Chan et al., 1996; Kim et al., 2005b). Animals were then backcrossed at least 7 times to a C57Bl6/J background for comprehensive *in vitro* and *in vivo* work. Animals were fed standard rodent chow and kept on a 12h-light/12h-dark reverse cycle, and were housed and cared for in accordance with the Canadian Council on Animal Care and CIHR ethics guidelines.

Cortical neuron culture:

Primary cortical neurons from mouse embryos were obtained as previously described (Xiang et al., 1996). Briefly, cortices from E14.5-E15.5 embryos (generated from a DJ-1^{+/-} x DJ-1^{+/-} or NRF2^{+/-} x NRF2^{+/-} breed) were isolated, dissociated and plated onto 6 or 24 well dishes. For survival assays, adenovirus was added to the cellular suspension at time of plating and the number of GFP positive cells in the culture was used as indicator of transduction efficiency at one Day *In Vitro* (1 DIV). Cells were then treated with MPP⁺ for indicated time and fixed with 4% PFA. Nuclei were visualized using Hoechst and cell survival was assessed as the percentage of alive (round with visible nucleoli) vs. total (alive + dead, dysmorphic, shrunken and picnotic) nuclei.

In vivo adenovirus delivery:

Mice of wild-type DJ-1^{-/-} or NRF2^{-/-} genotype were administered unilateral striatal injections of NRF2, DJ-1 or control (GFP) adenoviruses a week prior to MPTP administration. Briefly, 2 x 10⁷ Plaque Forming Units (PFU) of virus were injected into the left striatum as previously described (Mount et al., 2007). Seven days were allotted in order

for the virus to effectively retrotranslocate to the SNc and express the target protein at relatively physiological levels as previously described (Aleyasin et al., 2010).

MPTP administration:

8-10 week old mice on a C57Bl6/J background were given a sub chronic regimen of MPTP. Briefly, daily administration of 30mg/kg of MPTP dissolved in 0.9% saline or saline alone were given by intraperitoneal (IP) injection for five days. 14 days following the commencement of injections, animals were anaesthetized, transcardially perfused with 0.9% saline followed by 4% Paraformaldehyde (PFA) and brains were extracted for histology.

Immunohistochemistry and dopaminergic neuron survival assessment:

Following perfusion, brains were post-fixed for 24 hours in 4% PFA and dehydrated in 10% Sucrose for 3 days (2 changes a day) and frozen with CO₂ on day 5 post-perfusion. SNc and striatum cryosections (14 μ m) were obtained with a Microm HM500 cryostat. Sections were stained by the avidin-biotin complex (ABC) method for TH (1:10,000; mouse, Immunostar) and DAT (1:2,000; rat, Chemicon) overnight followed by Biotin (1:250) and streptavidin-HRP (1:250) conjugation and visualization through 3,3'-Diaminobenzidine (DAB) as previously described (Mount et al., 2007).

Co-Immunoprecipitation:

Transient transfections were carried out using Lipofectamine 2000 (Invitrogen) HEK-293 cells. Cells were seeded in 60mm dishes and co-transfected with 8 μ g of total plasmid DNA (V5-KEAP1, Flag-DJ-1, or control vectors), and harvested twenty-four hours post-transfection. When indicated cells were treated with H₂O₂ (300 μ M) for 5 minutes prior to harvesting. Samples were harvested in lysis buffer (25mM Tris-HCl [pH7.4], 150mM NaCl, 1mM EDTA, and 0.2% Triton X-100) supplemented with protease inhibitors (Roche).

Immunoprecipitations were performed through incubation of anti-V5 (Abcam) antibody overnight at 4°C, followed by incubation with anti-mouse Ig IP beads (eBiosciences) at 4°C for 1 hour. Beads were washed three times with lysis buffer without protease inhibitors. Samples were then subject to SDS-PAGE and Western Blot analysis.

Statistical analysis:

Statistical analysis was performed on samples to determine levels of significance. Students T-Test or One-way ANOVA followed by Tukey's Least Significant Difference *post-hoc* tests were used. All quantified data is presented as mean + standard error of the mean (S.E.M).

Acknowledgements:

We would like to acknowledge Dr. Yuet Wai Kan (University of Hong Kong) for the generation of NRF2 null mice and Dr. Jeffrey Johnson (University of Wisconsin-Madison) for use of viral constructs. This work was funded in part by grants from Parkinson Society Canada, the Canadian Institutes of Health Research, Heart and Stroke Foundation of Ontario, Neuroscience Canada (Brain Repair Grant), Parkinson's Disease Foundation, The Michael J. Fox Foundation for Parkinson's research, Parkinson Research Consortium, the Canadian Stroke Network, the Heart and Stroke Foundation Centre for Stroke Recovery and World Class University program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology, South Korea (R31-2008-000-20004-0) to D.S.P. D.S.P. is a recipient of the Heart and Stroke Foundation of Ontario Career Investigator Award. M.W.C.R is a recipient of the HSFC Focus on Stroke Award as well as the CIHR Training Program in Neurodegenerative Lipidomics Graduate Student Supplement Scholarship. P.C.M is a recipient of the PRC Toth Family Fellowship in Parkinson's Research. We would also like to thank Sarah J. Hewitt, Linda Jui and Carmen Estey for technical assistance and scientific input.

Chapter 4: Progressive Dopaminergic Cell Loss With Unilateral-to-Bilateral Progression in a Genetic Model of Parkinson's Disease.

*In revision: **Proceeding of the National Academy of Sciences USA***

Maxime Rousseaux, Paul C. Marcogliese, Dianbo Qu, Raymond H. Kim, Ruth S. Slack,
Diane C. Lagace, Tak W. Mak, David S. Park

Statement of Author contribution:

In this manuscript, we report a novel pre-clinical murine model of PD pathology that displays dopaminergic cell loss that recapitulates many traits observed in human PD. First, the degeneration is unilateral in origin and progresses to bilateral loss with aging. This finding is particularly exciting given the unilateral-to-bilateral progression of sporadic and familial PD. This phenotype arises in a subset of DJ-1 null animals fully backcrossed to a C57Bl6/J background. Second, this degeneration is also not reliant on the introduction of exogenous stressors but occurs in an age dependent fashion, another critical feature of human PD. Third, degeneration of DA neurons is concentrated within the SNc with limited degeneration in the VTA, another classic symptom of human PD. Fourth, we observe numerous indices of early compensation including exuberant fiber staining and excess post-synaptic activity, also known to occur in human PD. Finally, we not only observe degeneration of dopaminergic neurons in the *Substantia Nigra*, but also in another nucleus pathologically affected in PD: the *Locus Ceruleus*. These findings are particularly interesting as it is the first report demonstrating robust neuronal loss as a result of PD-related gene ablation. Accordingly, these findings reveal a new model where one may study the early indices and pathological progression of PD in a pre-clinical manner.

Maxime Rousseaux designed the experiments. **Maxime Rousseaux** performed the majority of the experiments with assistance from Paul C. Marcogliese and Dianbo Qu. Paul C. Marcogliese performed the majority of the behavior experiments. The project was supervised and guided by Dr. David S. Park. Dr. Raymond H. Kim from the University of Toronto has generated the original DJ-1 null mice. Diane C. Lagace provided the behavioral facilities in addition to scientific and technical input. **Maxime Rousseaux** and Dr. David

Park carried out data analysis. **Maxime Rousseaux** and Dr. David Park redacted the manuscript, with collaboration and scientific input from Dr. Ruth Slack and Dr. Tak W. Mak.

Classification: BIOLOGICAL SCIENCES, Neuroscience

***Progressive Dopaminergic Cell Loss With Unilateral-to-Bilateral
Progression in a Genetic Model of Parkinson's Disease.***

Maxime Rousseaux^{*}, Paul C. Marcogliese^{*}, Dianbo Qu^{*}, Raymond H. Kim[†], Ruth S. Slack^{*},
Diane C. Lagace^{*}, Tak W. Mak[†], David S. Park^{*,‡,§}

Number of text pages: 19

Number of figures: 5

Abstract:

DJ-1 mutations cause autosomal recessive early-onset Parkinson's disease (PD). We report a novel model of PD pathology: the DJ1-C57 mouse. A subset of DJ-1 nullizygous mice, when fully backcrossed to a C57Bl/6J background display dramatic early-onset unilateral loss of dopaminergic neurons in their Substantia Nigra *pars compacta*, progressing to bilateral degeneration of the nigrostriatal axis with aging. In addition, these mice exhibit age-dependent bilateral degeneration at the level of the *Locus ceruleus*. These findings effectively recapitulate the early stages of PD. Therefore, the DJ1-C57 mouse provides a potentially effective tool to study the pre-clinical aspects of neurodegeneration in a relevant manner to better develop effective therapeutics.

Introduction:

Parkinson's disease (PD) is a progressive neurodegenerative disorder with complex symptomology and etiology affecting an ever-increasing number of individuals. Though multifactorial in nature, increasing insight has been granted with regards to the pathogenesis of the disease through investigation of genes linked to PD. Surprisingly, despite the number of genetically manipulated mice created in attempts to recapitulate the disease process, little or none have shown clear or robust neurodegeneration specific to the Substantia Nigra *pars compacta* (SNc) (Reviewed in (Dawson et al., 2010)). For instance, loss-of-function mutations in the *DJ-1* (*PARK7*) gene cause early-onset autosomal recessive PD (Bonifati et al., 2003; Hague et al., 2003). However, generation of DJ-1 nullizygous mice (*DJ-1^{-/-}*) by various laboratories including our own failed to detect any basal levels of neurodegeneration even in aged mice (Andres-Mateos et al., 2007; Chen et al., 2005; Goldberg et al., 2005; Kim et al., 2005b). Therefore, the creation of murine PD models that demonstrate significant DA loss remains an acute need in the field. This is particularly critical if we are to understand how specific signaling pathways govern DA loss in human PD. Presently, most mechanistic studies of DA loss rely on acute toxin models of Parkinsonism. However, the relevance of such studies to the human condition remains uncertain for obvious reasons. This potential discrepancy is highlighted by a number of failed clinical trials that have heavily relied on toxin models as pre-clinical evidence for efficacy (Marks et al., 2010; Parkinson Study Group, 2007; Snow et al., 2010; Waldmeier et al., 2006). A more representative model of DA loss which utilizes known factors in human PD is likely important.

Results:

In the course of our studies examining the effects of environmental perturbations in DJ-1^{-/-} mice, we continued to examine the long-term effects of DJ-1 deficiency on dopaminergic neuron loss. Importantly, this was accomplished in animals completely backcrossed onto a C57Bl/6J background (14x backcrossed, DJ-1^{-/-} herein referred to as DJ1-C57). Intriguingly, unilateral SNc degeneration in a subset of these DJ1-C57 knockout mice is observed as early as 2 months of age (Fig. 4.1A, Fig. 4.S1, and Supplementary Table 4.1). This phenotype is not observed in animals younger than 2 months ($n = 8$; fig. 4.S1) thus indicating that this defect is unlikely to be developmental in origin. Moreover, this phenotype is not observed in any of the wild type mice examined ($n = 71$). In addition, the ventral tegmental area (VTA) of these mice is mostly spared (Fig. 4.1D). This latter finding is particularly interesting given the observation that in *post-mortem* brains from PD patients, VTA neurons remain relatively protected compared to their nigral counterparts (Perl, 2011).

Figure. 4.1: Young affected DJ1-C57 mice exhibit selective unilateral degeneration in

their SNc. (A) Representative midbrain sections of DJ1-C57 Affected (top), DJ1-C57 Unaffected (middle) and wild-type (bottom, WT) mice depicting TH staining in the SNc and VTA. (B) Quantification of (A) by stereology of total number of TH-positive cells in the SNc or (C) of cresyl-violet stained cells at the level of the medial terminal nucleus (MTN) in the SNc. (D) Quantification of TH-positive neurons in the VTA of WT and DJ-1 Affected mice. Note that wild-type, DJ1-C57 affected and DJ1-C57 Unaffected are represented by blue, red and yellow bars, respectively. Side A is depicted as solid shading and Side B as hatched shading. NS and *** denote $p > 0.05$ and $p < 0.001$, respectively, ANOVA followed by Tukey's LSD *post-hoc* tests. Data are represented as means ($n = 7-80$ per group) $\pm$ s.e.m.

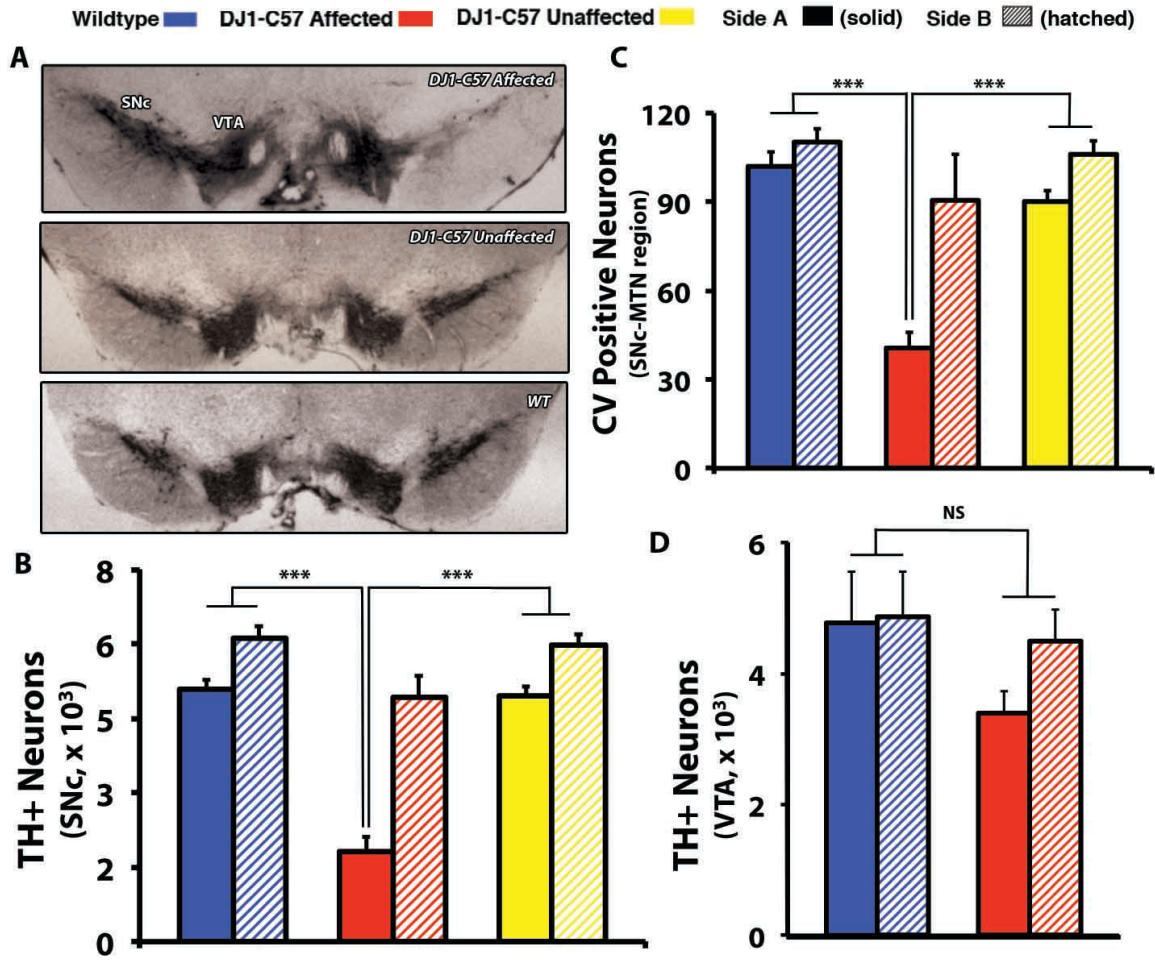
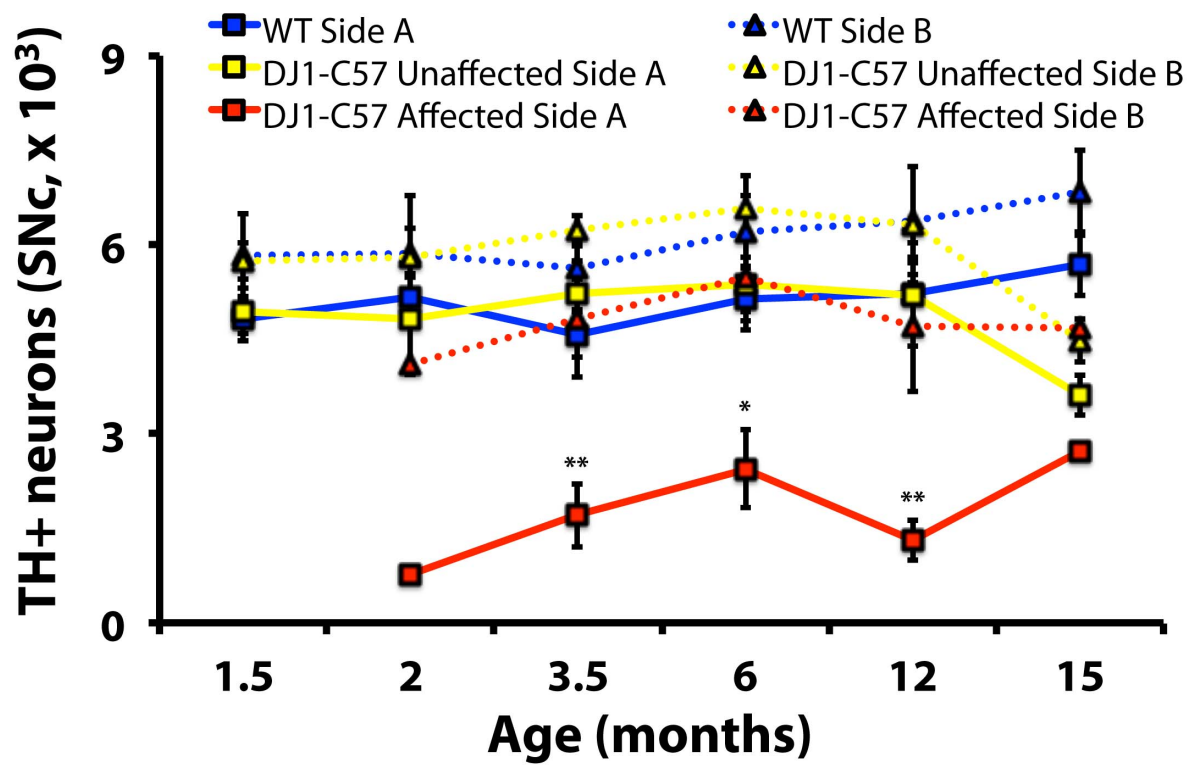


Figure 4.S1: Unilateral dopaminergic cell loss in the SNc of a subset of DJ1-C57 mice as early as 2 months of age. Stereological counts of TH-positive neurons in midbrain sections from WT and DJ1-C57 mice were performed at various time points. No mice were qualified as “affected” according to the >40% unilateral criteria in 6 week old mice. Each data point represents mean $\pm$ s.e.m. $n = 1-37$ per data point. ** denotes $p < 0.01$ by ANOVA followed by Tukey’s LSD *post-hoc* test.



Supplementary Table 4.1: Penetrance of “Affected” phenotype over time in DJ1-C57

and WT mice. Penetrance was measured as an animal possessing a greater than 40% loss of neurons on one side of the SNc (Side A) vs. the other side (Side B). Samples were gathered at 1.5, 2, 3.5, 6 12 and 15 months. Sample sizes (*n*) for each group varied from 5-37. Only DJ1-C57 animals exhibited the phenotype which begins at 2 months of age.

Genotype	Age (Months)	Sample size (n)	Unilateral phenotype?	Penetrance (%)
WT	1.5	5	N	0
	2	9	N	0
	3.5	19	N	0
	6	16	N	0
	12	10	N	0
	15	12	N	0
total WT	-	71	N	0
DJ1-C57	1.5	8	N	0
	2	8	Y	12.5
	3.5	37	Y	16.2
	6	23	Y	17.4
	12	7	Y	42.9
	15	13	Y	7.7
total DJ1-C57	-	96	Y	15.6

n=15 penetrant DJ1-C57 vs n=0 penetrant wt

In order to objectively assess this phenotype, mice in this study are classified as either “affected” (unilateral phenotype: having a greater than 40% unilateral reduction of dopaminergic cells in the SNc when compared to the other side) or “unaffected” (no unilateral phenotype: having similar bilateral dopaminergic cell numbers). No clear side or sex specificity is observed (Right, 53%; Female, 67%, respectively). Thus, in order to maintain consistency, “Side A” is the term given for the side of the brain with the least number of neurons, regardless of the genotype (Side B being the side with more dopaminergic neurons). When quantified, affected DJ1-C57 mice exhibit a dramatic reduction of neurons in their SNc as visualized by tyrosine hydroxylase (TH) and cresyl violet (CV) staining (Fig. 4.1*B, C* and Fig. 4.3*A*). Upon closer magnification of the SNc, the affected DJ1-C57 mice exhibit TH-positive fiber staining but with clear neuronal process disruption. When quantified, the remaining fibers at the level of the SNc in the affected DJ1-C57 mice display elevated number of shortened processes with obvious neuritic beading (Fig. 4.2*A*) when compared with unaffected DJ1-C57 or control mice. Consistent with this finding, an increase in microgliosis is noted in young, affected animals (Fig. 4.3*B*), whereas no clear increase in astrocytosis to the lesion site is observed (Fig. 4.S2).

Figure. 4.2: Widespread process disruption and aberrant striatal innervation in

young affected DJ1-C57 mice. (A) Fiber sprouting in WT (top left panel) and DJ1-C57 Affected animals (top right panel). Distribution of quantified uninterrupted process (TH+) length in a single vision plain (in μm) is presented on the bottom panel. (B) Representative sections of striatum stained for ΔFosB in young WT (left panel) and DJ1-C57 Affected (middle panel) mice. Right panel depicts quantification of ΔFosB -positive puncta in the striatum. (C) Representative sections of the striatum stained for TH as in (B). Quantification of striatal TH density performed in the right panel. Wild-type, DJ1-C57 affected and DJ1-C57 Unaffected are represented by blue, red and yellow bars, respectively. Side A is depicted as solid shading and Side B as hatched shading. NS, * and ** denote $p > 0.05$, $p < 0.05$ and $p < 0.01$, respectively, ANOVA followed by Tukey's LSD *post-hoc* tests. Data are represented as means ($n = 3-11$ per group) $\pm$ s.e.m.

Wildtype ■ DJ1-C57 Affected ■ DJ1-C57 Unaffected ■ Side A ■ (solid) Side B ▨ (hatched)

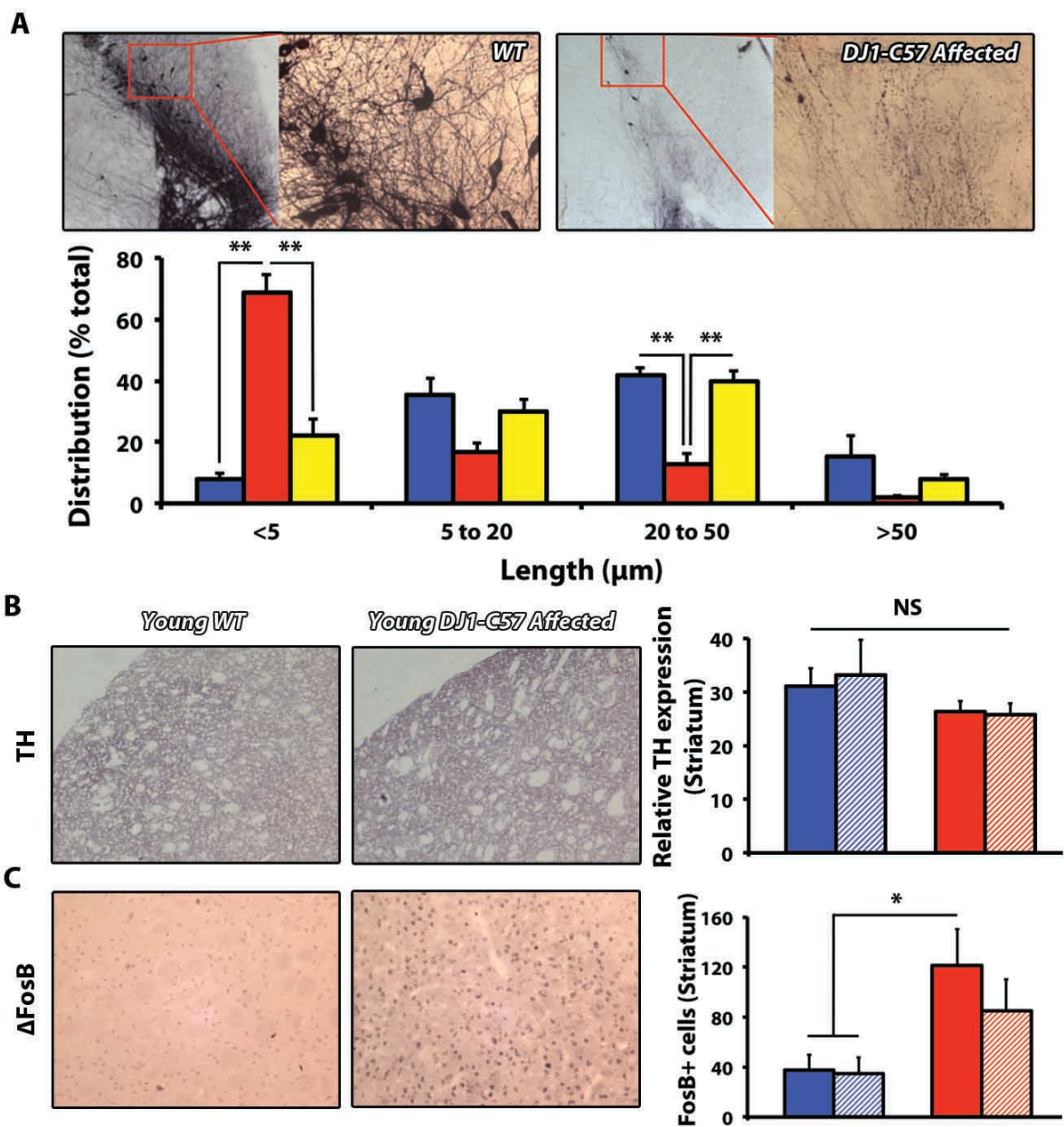


Figure 4.3: Focal microgliosis in young affected DJ1-C57 mice. (A) Representative midbrain sections stained for cresyl violet (CV) in young wild-type and DJ1-C57 affected mice (1000x magnification). Thin arrows denote typical morphology of dopaminergic neurons of the SNc, thick arrows denote shrunken, dead nuclei and arrowheads denote appearance of cells with altered morphology. (B) CD11b staining in the midbrain of young wild-type (left panel) and DJ1-C57 affected (right panel) mice was quantified and represented as the number of Cd11b positive cells in the medial terminal nucleus region of the SNc. Wild-type and DJ1-C57 affected are represented by blue and red bars, respectively. Side A is depicted as solid shading and Side B as hatched shading. NS and * denote $p > 0.05$, $p < 0.05$, respectively, ANOVA followed by Tukey's LSD *post-hoc* tests. Data are represented as means ($n = 3-9$ per group) $\pm$ s.e.m.

Wildtype  DJ1-C57 Affected  Side A  (solid) Side B  (hatched)

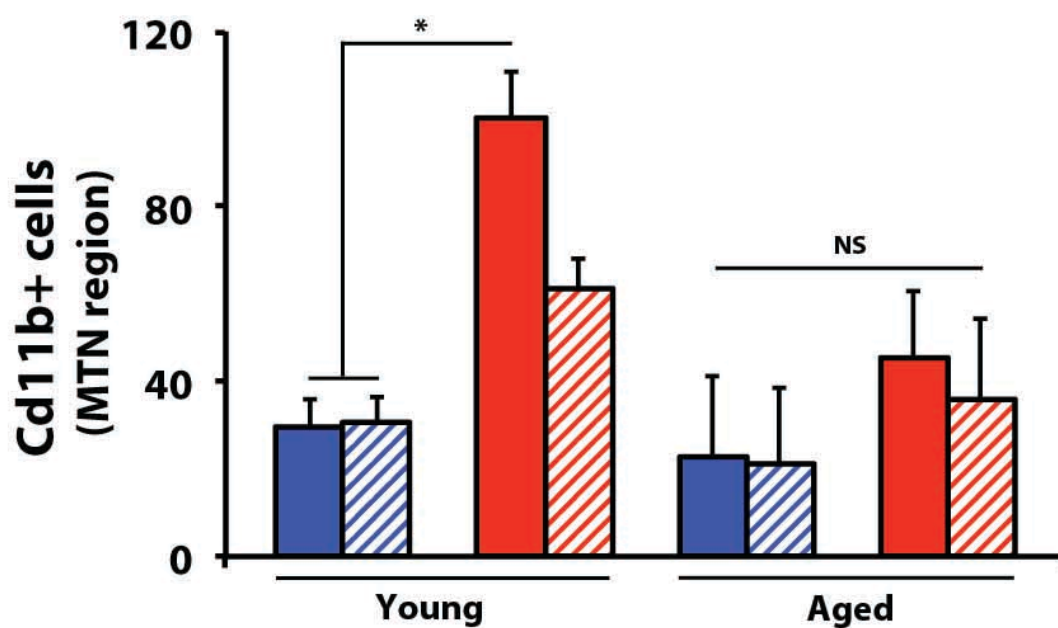
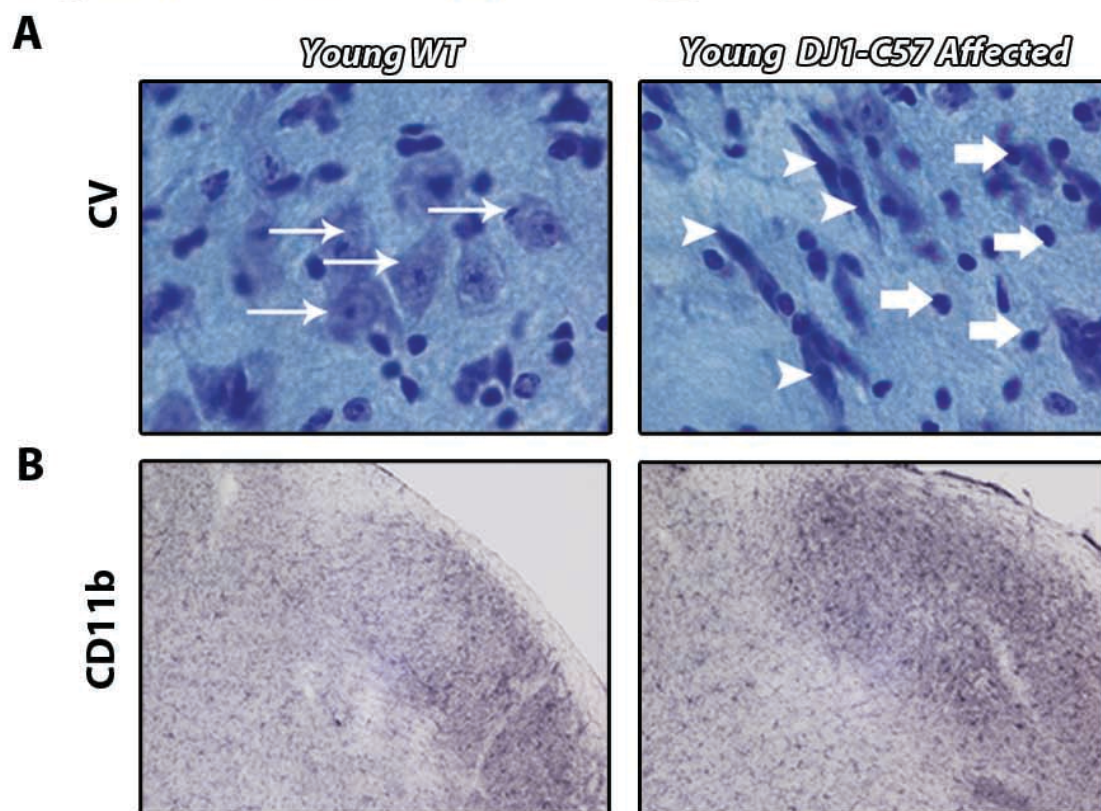
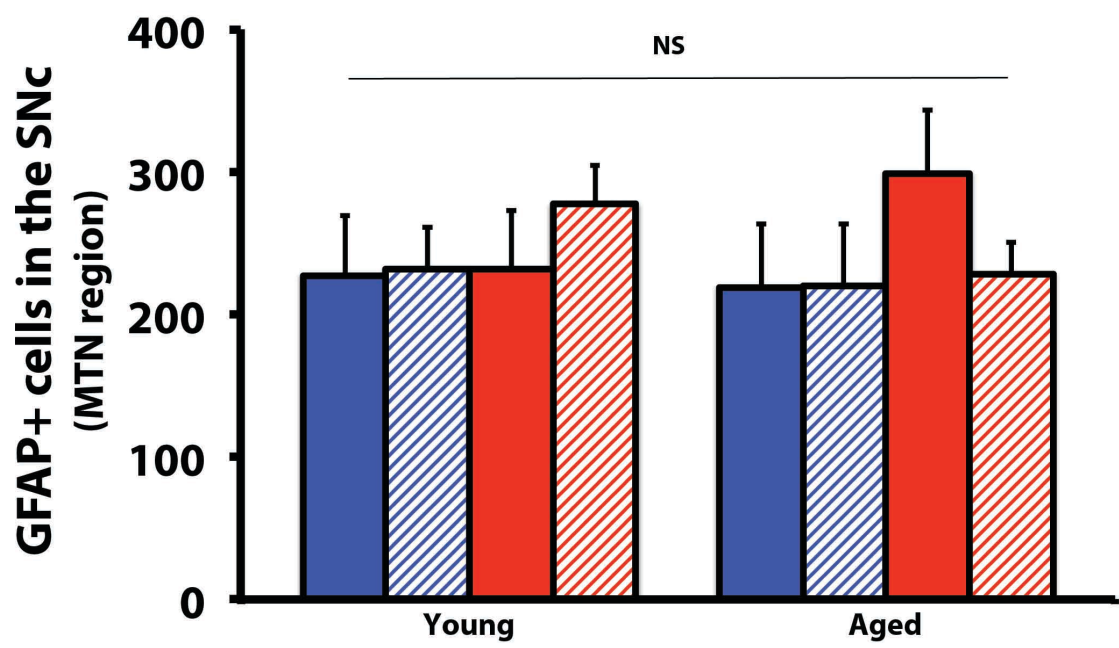
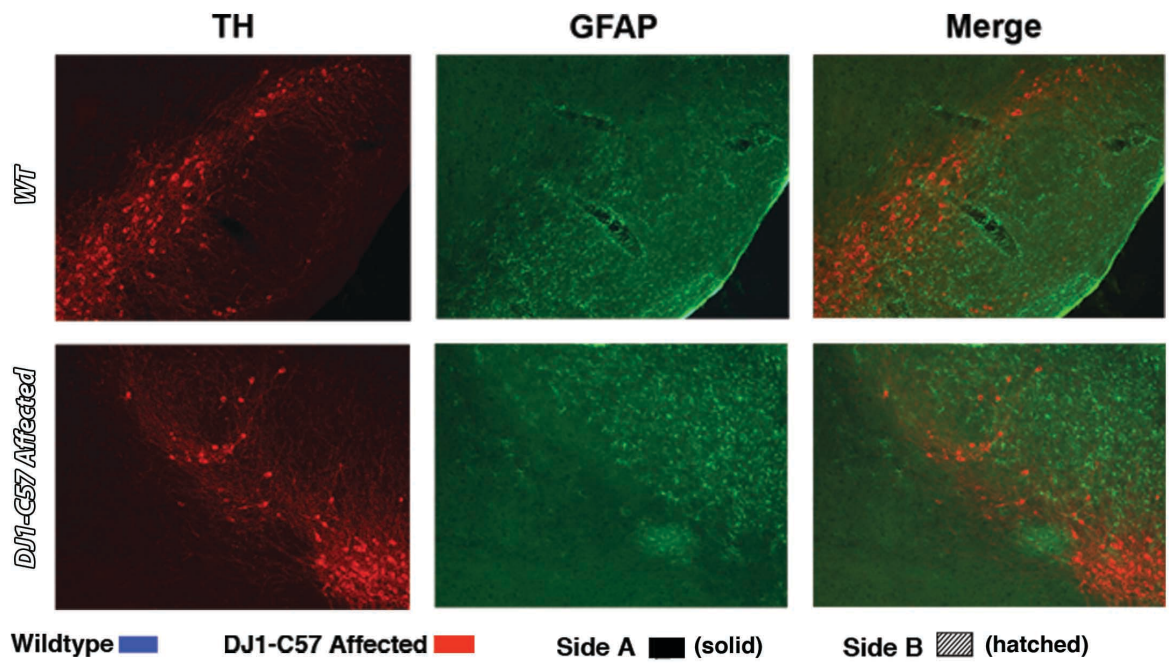


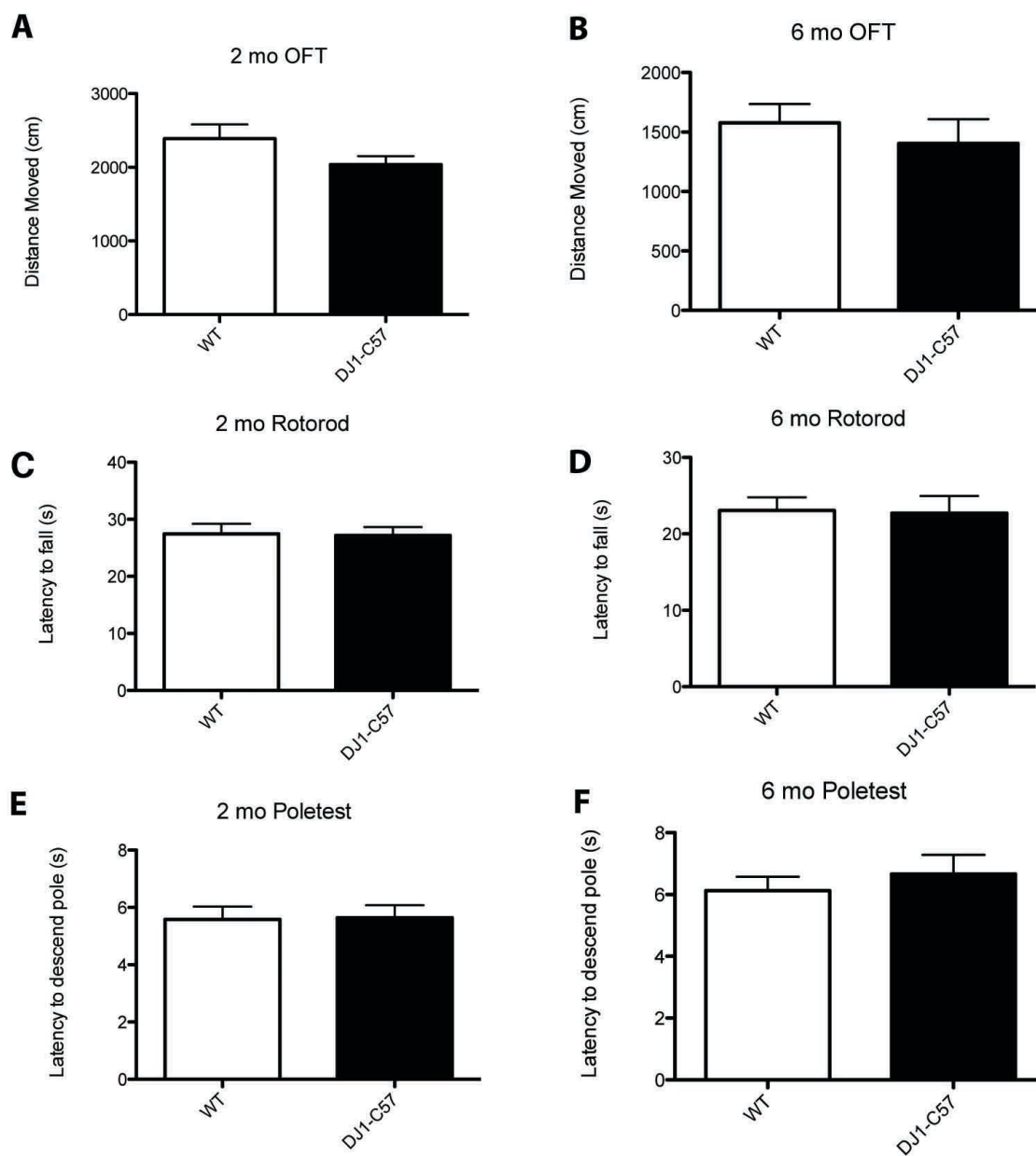
Figure 4.S2: No visible astrocytic aggregation in affected DJ1-C57 midbrain. (Top panel) Midbrain sections from young wild-type ($n = 6$) and DJ1-C57 affected ($n = 6$), left; or aged wild-type ($n = 4$) and DJ1-C57 mice ($n = 4$), right, were co-stained for GFAP and TH. (Bottom panel) GFAP positive puncta in the SNc (medial terminal nucleus region) were quantified in 3 representative sections. NS denotes a non-significant difference by means of ANOVA.



To assess whether this histopathological phenotype corresponds with a functional outcome, we subjected animals to behavioral testing. However, DJ1-C57 affected mice do not exhibit a clear decrease in motor neurological score at 2 or 6 months of age (Fig. 4.S3). The lack of behavioral differences may be accounted by two observations. First, examination of the striatal DA terminals reveals no clear loss in striatal fibers in young animals (Fig. 4.2B). This finding is supported by the possibility that the sprouting of dysmorphic neurites (as seen in Fig. 4.2A) from the SNc may be compensating for the loss of cell bodies as previously reported (Pritzel et al., 1983; Song and Haber, 2000). Second, an increase in the striatal post-synaptic marker Δ FosB is observed in affected DJ1-C57 mice (Fig. 4.2C). PD patients have been shown to have upregulated Δ FosB in their caudate/putamen (Tekumalla et al., 2001). Moreover, Δ FosB has previously been shown to be upregulated in toxin models of neurodegeneration such as MPTP and 6-OHDA as a compensatory response to a loss of dopaminergic innervation (Doucet et al., 1996; Perez-Otano et al., 1998).

Figure 4.S3: No visible motor behavior defects in young DJ1-C57 mice. Littermate DJ1-

C57 mice ($n = 15$) and WT ($n = 12$) controls were subjected to motor behavioral testing at 2 months (A, C, E) and 6 months (B, D, F). (A, B) Open field test was performed for 10 minutes and the distance travelled was measured (cm). (C, D) Rotorod test was performed and latency to fall was measured (in seconds, s). (E, F). Bars represent means $\pm$ s.e.m. No significant differences were observed by ANOVA.



With aging of DJ-1 deficient animals, an increase in the prevalence of the DJ1-C57 “affected” unilateral phenotype is observed over time, peaking at 12 months of age (42.9% penetrance, Fig. 4.4A and Supplementary Table 1). When only affected unilateral DJ1-C57 animals are considered, there is a clear loss in *total* number of SNc DA neurons even at early time points (fig. 4.S4). However, if *all* (affected and unaffected) DJ-1 deficient animals are evaluated together, the total number of dopamine neurons is not significantly reduced until later aging stages (15 months). At this time, the unilateral phenotype dissipates and a more bilateral loss phenotype is observed (Fig. 4.4A,B and Supplementary Table 1). Interestingly, these aged mice, unlike at the earlier times, exhibit a decrease of DA staining in their striatal terminals when assessed for TH (Fig. 4.4C).

Figure 4.S4: Bilateral dopaminergic cell loss in the SNc of aged DJ1-C57 mice.

Stereological counts of TH-positive neurons in midbrain sections from WT and DJ1-C57 mice were performed at various time points. Each data point represents mean $\pm$ s.e.m. $n = 1-37$ per data point. * and ** denote $p < 0.05$ and $p < 0.01$, respectively, by ANOVA followed by Tukey's LSD *post-hoc* test.

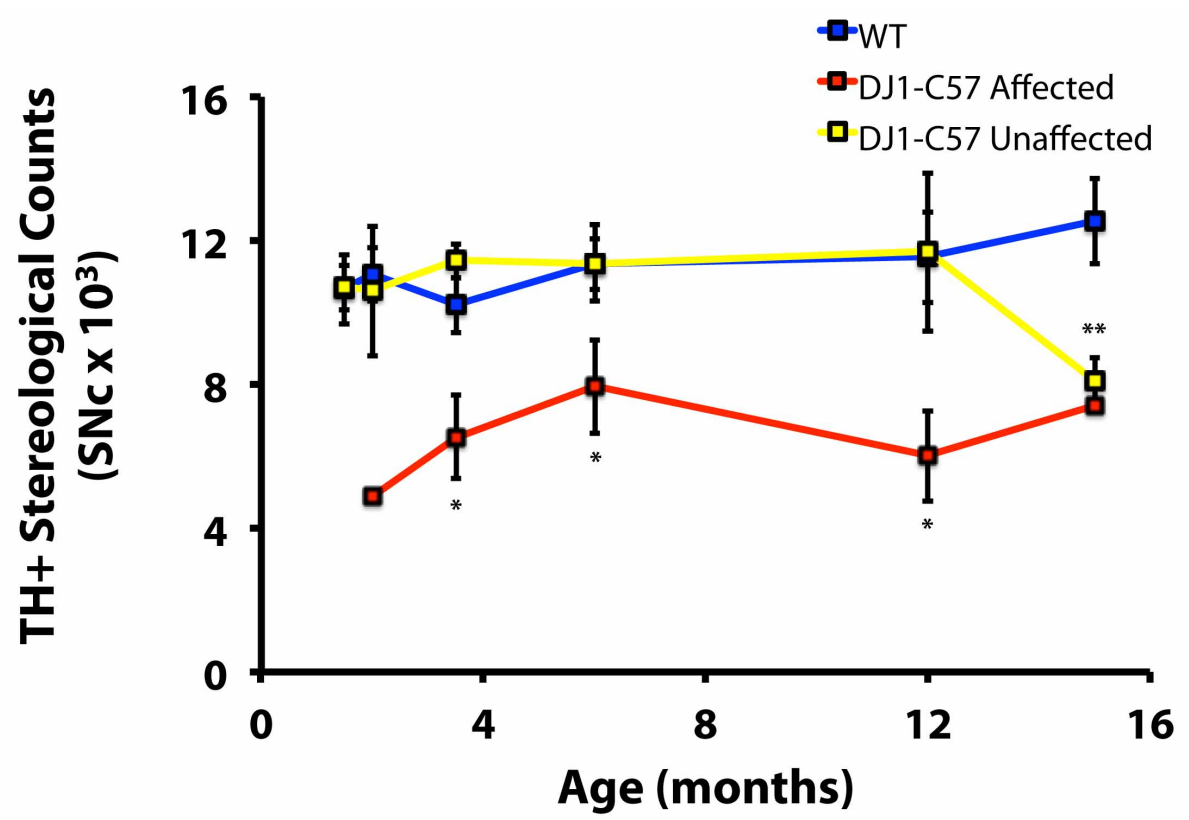


Figure 4.4: Aged DJ1-C57 mice exhibit bilateral dopaminergic and noradrenergic

denervation in the brain stem. (A) Penetrance of unilateral phenotype over time.

Penetrant threshold: greater than 40% loss of dopaminergic neurons on one side (Side

A) versus the other (Side B). (B) Total stereological counts of dopaminergic neurons in

the SNc of WT and DJ1-C57 aged (15 months) animals. (C) Representative striatal

sections of aged (12-15 months) WT (top left panel) and DJ1-C57 (bottom left panel)

mice stained for TH. Quantification of TH expression in the striatum relative to the

corpus callosum was performed in young and aged animals (right panel). (D)

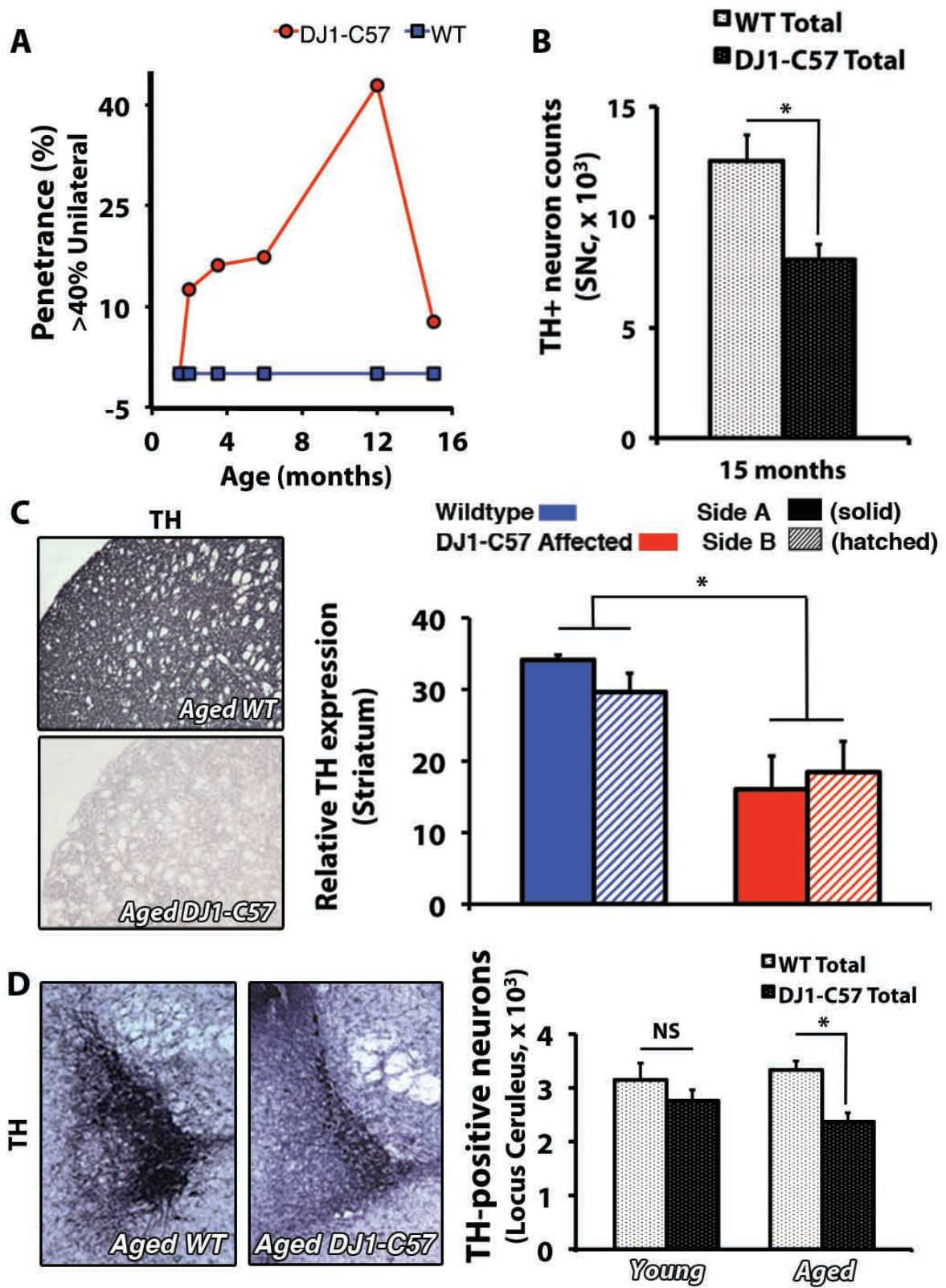
Representative micrographs of *locus ceruleus* sections in the pons stained for TH for

either aged WT (left panel) or aged DJ1-C57 (middle panel). Quantification of TH-

positive neurons is performed for both young and aged animals in the right panel. NS

and * denote $p > 0.05$ and $p < 0.05$, respectively, ANOVA followed by Tukey's LSD

post-hoc tests. Data are represented as means ($n = 3-13$ per group) $\pm$ s.e.m.



Finally, it has been reported that PD patients exhibit degeneration not only in the SNc but also in other nuclei of the brainstem including the *locus ceruleus* (LC) (Forno and Alvord, 1974). Therefore, LC of DJ1-C57 mice were examined for TH-immunoreactive cell bodies. A significant reduction in noradrenergic cells of the LC was observed in aged DJ1-C57 mice compared to WT controls (Fig. 4.4D).

Discussion:

We have demonstrated a robust and progressive pre-clinical genetic PD phenotype in a subset of the DJ1-C57 mice. In backcrossing and extensively interbreeding these mice, we obtained a subset of DJ1-C57 mice that exhibit dramatic unilateral nigral degeneration as early as 8 weeks of age – a finding potentially consistent with the early-onset pathogenicity of human DJ-1 mutations (Bonifati et al., 2003). This cell loss is accompanied with compensatory sprouting of dysmorphic and beading neurites as well as microgliosis, a result congruent with the notion that microglia may induce neuritic beading during neuronal dysfunction (Takeuchi et al., 2005). Furthermore, findings of compensatory sprouting upon cell loss; dysmorphic neurites as well as increases in pro-inflammatory response are all present in *post-mortem* samples from PD patients (Greenwood et al., 1991; McGeer et al., 1988; Mount et al., 2007; Nagatsu and Sawada, 2005; Ouchi et al., 2005; Whitton, 2007). Therefore, given the early age of onset of degeneration of these mice, these findings are of particular significance and may correlate with early stages of PD.

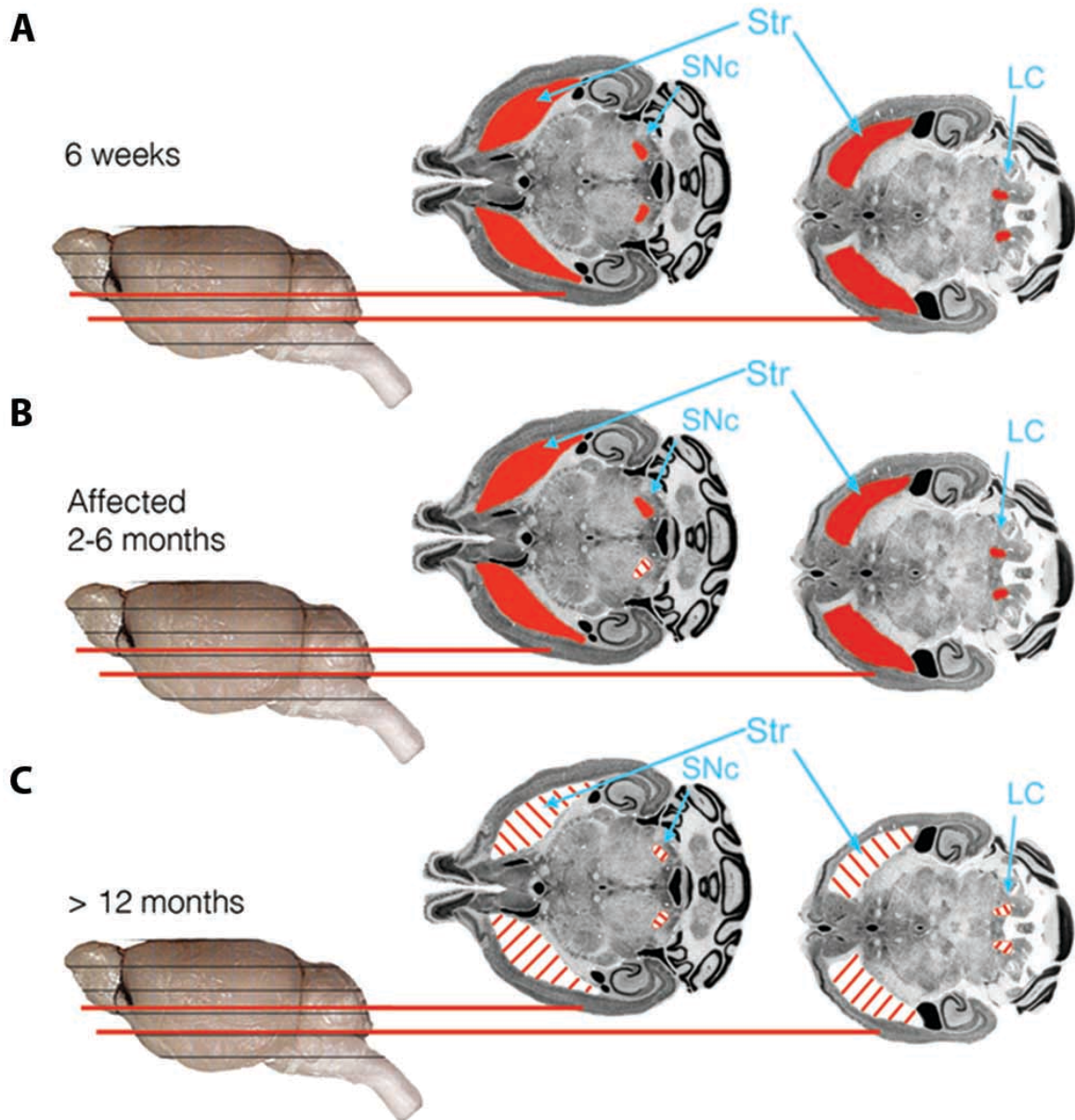
We also note here that while DJ-1 loss-mediated motor phenotypes are apparent in human patients, this might not occur in mice with a relatively short time life span such as our DJ1-C57 mice. However, the earliest pathological changes associated with this genetic form of PD are nonetheless clearly prominent. Thus, much like pre-clinical PD where no clear clinicopathological correlate is apparent, a compensatory mechanism such as neuritic sprouting or post-synaptic sensitization may account for the lack of motor defects in these young animals. Furthermore, the relevance of this model is made even more apparent as the DJ1-C57 mice age. Aged DJ1-C57 mice show bilateral degeneration of nuclei in their SNc as well as their projections to the striatum. In addition, these mice exhibit cell loss at the level

of the *locus ceruleus*, a pathological characteristic of the human condition. Additionally, the above-mentioned pathological findings are of particular interest given that no *post-mortem* analyses of human DJ-1 mutant-carrying patients have been reported. In this regard, the present DJ-1 mouse model presents a potentially important role in filling this gap in our understanding of DJ-1 mediated PD.

Collectively, we present a novel murine model that reproduces a clinically prominent phenotype with the modification of a PD-related gene. Affected DJ1-C57 mice display: 1) Unilateral dopaminergic cell loss that is preferential to the SNc versus VTA as early as 2 months of age; 2) aberrant neuritic processes with microgliosis in the SNc and increased Δ FosB staining in the striatum at a young age; and 3) progression to bilateral degeneration of the nigro-striatal axis as well as the *locus ceruleus* at an older age (Model, Fig. 4.5), a finding particularly interesting given the typical unilateral-to-bilateral progression of the disease (Pahwa R, 2003). This model thus provides a tool to elucidate the first pathogenic changes occurring in PD as well as the mechanisms of its progressive development in an unprecedented fashion.

Figure 4.5: DJ1-C57 pre-clinical model of dopaminergic neurodegeneration. (A)

Altered representative micrographs taken from the Mouse Brain Library (Rosen et al., 2003) (Rosen *et al.*, 2003; www.mbl.org) depicting healthy (red shaded) SNc, Striatum and LC in 6 week old DJ1-C57 mice or all WT groups examined. (B) Affected DJ1-C57 mice demonstrate unilateral dopaminergic cell loss in the SNc but not in the LC. (C) Aged DJ1-C57 mice exhibit widespread degeneration in their nigro-striatal tract as well as their *locus ceruleus*.



The reason behind this significant phenotype is unknown. Previous studies, including work from our own lab, have established a role for DJ-1 in the management of oxidative stress and mitochondrial dynamics (Aleyasin et al., 2010; Andres-Mateos et al., 2007; Canet-Aviles et al., 2004; Irrcher et al., 2010; Martinat et al., 2004). This latter role is of interest since mitochondria are a major source of ROS. *In vivo*, this may be due to an increase in mitochondrial oxidant stress in dopaminergic neurons from DJ-1 null mice stemming from compromised mitochondrial uncoupling through down-regulated UCP proteins (Guzman et al., 2010). How these factors impact on our phenotype is unclear. It will be important to establish the modifying factors which interact with DJ-1 deficiency that gives rise to the phenotype in the future.

Experimental Procedures:

DJ1-C57 mouse creation: DJ1^{-/-} mice were generated as previously described (Kim et al., 2005b). Mice were subsequently backcrossed 14 times onto a pure C57Bl/6J background (Charles River) to obtain DJ1-C57 mice. Animals were then interbred extensively for colony maintenance and experimentation. Animals were kept at 25 °C on a light (12 h) / dark (12 h) cycle with *ad libitum* access to standard rodent laboratory chow and water. Animal care was carried out in accordance with the guidelines of the Canadian Council and Care of Animals in Research and the Canadian Institutes of Health Research and was approved by the University of Ottawa Animal Care Veterinary Services.

Histology: After being perfused transcardially, mice brains were fixed in 4% paraformaldehyde and cryoprotected as described elsewhere (Crocker et al., 2001). Midbrain sections containing the SNc (40 µm), pontine sections containing LC (40 µm) and striatal (14 µm) sections were immunostained via avidin-biotin complex staining as previously described (Mount et al., 2007).

Dopaminergic cell survival quantification: Dopaminergic neuron survival in the SNc was blindly assessed by stereology using Stereo Investigator as previously described (Mount et al., 2007). Striatal TH quantification was performed at 200X. For each picture, five samples of striatum and one sample of corpus callosum were used for densitometric analysis. Relative intensity of immunodetection was calculated using NIH ImageJ v.1.41o. For each sample three slices of striatum were used to calculate the mean striatal density.

Neuritic beading measurement: Neuritic beading was measured using ImageJ. Briefly, average length of uninterrupted process in a visually focused plane was measured as 20

measurements/section and measuring 3 sections per animal. Raw data was then binned into 5 categories of length and represented as % distribution.

Δ FosB and CD11b measurement: Striatal (Δ FosB) and midbrain (CD11b) sections were stained and 3 pictures were taken per animal, per side. Puncta in a given visual field were blindly assessed using ImageJ v1.41o.

Locus Ceruleus neuron quantification: Noradrenergic cell survival in the LC was measured by counting 4 representative sections and projecting their counts to a total value as previously described (German et al., 2000).

Cresyl violet quantification: Cresyl violet staining and quantification was performed as previously described (Mount et al., 2007). Briefly, cell viability in the MTN region of the midbrain was assessed as per the nuclear integrity of the cells present.

Antibodies used: CD11b (1:200; AbD Serotec), FosB (1:250; Santa Cruz Biotechnologies), GFAP (1:1000; Cell Signaling), and TH (1:10,000; Immunostar or 1:2,000; Chemicon) were used for either avidin-biotin complex (ABC) visualization by 3,3'-diaminobenzidine (DAB) or via fluorophore-conjugated secondary antibody.

Statistical Analysis: Data throughout the paper are expressed as average $\pm$ s.e.m for a given sample size (n). Statistical analysis for histological and behavioral data was performed by means of either a paired t-test or one-way ANOVA followed by Tukey's least significant difference (LSD) *post-hoc* test as indicated in the text and figure legends.

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Author contributions: M.W.C.R performed most of the experiments, interpreted the results and wrote the manuscript. P.C.M. performed most of the behavioral analysis of the mice and edited the manuscript. R.H.K generated the original DJ-1 null mouse. D.Q. offered advice on project planning and data interpretation. D.C.L. offered advice and facilities for behavioral analysis. R.S.S and T.W.M. provided advice on project planning. D.S.P directed, supervised the project and edited the manuscript.

Competing interests: The authors declare no competing financial interests.

Supplementary Information:

Supplemental Methods: Behavior: In all behavior tests except for the pole test, mice were habituated to the testing room with white noise for an hour before testing. In the Open Field Test (OFT), mice were placed in the corner of a novel ($\sim 45 \text{ cm}^3$) box and video analyzed for ten minutes. Total distance moved was measured in centimeters (cm). In the Rotorod test, mice were placed on an accelerating Rotorod and latency to fall of the rod was measured in seconds. In the Pole Test, mice were placed atop an 18" pole and time to descent was measured in seconds. For aged animals, Beam Break, Grid, Elevated Plus testing was also performed. Briefly, mice were subjected to a novel cage for 24 hours and total activity (beam breaks) was recorded. For the Grid test, mice were placed on an 8" x 10" grid (0.5 cm spacing) and grid was flipped over for a maximum of 60 seconds. Latency to fall from the grid was measured in 3 repeated trials. For the Elevated Plus test, mice were placed in the closed arm of the elevated plus and allowed 10 minutes to freely explore. Percentage of time in open vs. closed arms was measured as well as total distance moved (cm). All video data was obtained and analyzed with the Ethnovision 8.0 software. Motor neurological scores (composite scores) were obtained as follows: each behavioral test was assigned a rank from 0 to 9 and the sum of all "points" for each behavioral test was calculated for a total score per mouse. For instance, a mouse staying on the Rotorod for 9 seconds received a score of 1 whereas a mouse staying on the Rotorod for 33 seconds received a score of 7.

Chapter 5:

General Discussion and Conclusion

Summary

Parkinson's disease is the second most common neurodegenerative disease affecting an increasing number of people in North America's aging population. Given the current clinical focus on symptomatic relief and not disease cessation or reversal, much of the basic research in the field of PD has focused on understanding the etiology of the disease to better create neuroprotective therapies.

The central topic of this thesis is focused on the mechanism of action of DJ-1. Specifically, we addressed its function with regards to the handling of oxidative stress in order to serve its pro-survival role within the cell. DJ-1 was first identified in 1997 as an oncogene and has since been given multiple roles including chaperone function (Shendelman et al., 2004), cell death inhibitor (Junn et al., 2005; Shinbo et al., 2005), translational regulator (van der Brug et al., 2008), signaling molecule (McNally et al., 2011) and antioxidant (Andres-Mateos et al., 2007). These putative cellular roles have been studied for many years in the hope that a defined pathway involving DJ-1 may be fully elucidated. The function of DJ-1 has additionally been underscored in 2003 when loss of function mutations in this gene were linked to early-onset PD (Bonifati et al., 2003). In fact, the generation of DJ-1 nullizygous mice has aided in the understanding of the role of DJ-1 within the cell though it has fallen short of serving as a fully representative model of the disease. For instance, DJ-1 has been shown to interact both functionally and biochemically with other PD genes such as PINK1, Parkin, α -synuclein and LRRK2; a notion giving credence to the complexity of the genetic contribution in this multifactorial disease (Meulener et al., 2005b; Moore et al., 2005; Venderova et al., 2009; Xiong et al., 2009). Moreover, DJ-1 has been linked to autophagy and mitochondrial dynamics and function (Guzman et al., 2010; Hao et

al., 2010; Irrcher et al., 2010; Thomas et al., 2011). This finding is particularly interesting given the extensive link between mitochondria in PD as previously alluded to in this thesis. Taken together, much insight has been provided with regards to cellular processes and pathways that DJ-1 takes place in. Nevertheless, the precise mechanism through which it acts to promote neuroprotection remains elusive. To this end, this thesis endeavors to elucidate the function of DJ-1 by:

1. Using the association of DJ-1 and PD as well as culminating evidence that oxidative stress is important in the pathogenesis of PD to generate pathways through which the PD gene DJ-1 may work; and
2. It seeks to generate an animal model of PD to better recapitulate the cardinal features of the disease.

It is by taking this two-tiered approach that we hope to elucidate the cellular and molecular mechanisms underlying to death of dopaminergic cells giving rise to this terrible disease.

Principal Findings

In this dissertation, we identified a more clear mechanism through which DJ-1 protects the cell against oxidative insult and without which results in dopaminergic cell death in humans. The results we presented in chapter two indicate that DJ-1 likely mediates its pro-survival, antioxidant effects through the pro-survival molecule AKT.

In summary, insult-mediated AKT activation is dramatically reduced in the absence of DJ-1. DJ-1 requires AKT to confer its neuroprotective role and, conversely, AKT requires the cellular presence of DJ-1 to be neuroprotective. Interestingly, we observed that AKT activation through DJ-1 likely happens through its recruitment to the plasma membrane

where it can bind to PIP3 and get phosphorylated – and thus activated – by PDK1 and mTORC2. These findings suggest that DJ-1 can facilitate the membrane recruitment of AKT, yet the mechanism is unclear. Evidently, better elucidation of a mechanism by which DJ-1 impacts AKT recruitment might give rise to better therapeutic targets and provide a better understanding of this neuroprotective pathway.

To further elucidate how the DJ-1-AKT axis mediates neuroprotection from oxidative stress, chapter three of this thesis addresses the role of the master antioxidant regulator NRF2 in DJ-1-mediated neuroprotection. In this study, we established the pre-existing AKT-NRF2 link in our model system by showing that pre-treatment of the pharmacological AKT inhibitor results in complete inhibition of the neuroprotective function of NRF2. Next, we noted that NRF2 overexpression could rescue the hypersensitivity to oxidative stress seen in DJ-1 knockout neurons both *in vitro* and *in vivo* models of PD. Importantly, we tested whether NRF2 acts downstream of DJ-1 and noted that DJ-1 overexpression could not rescue the hypersensitivity to ROS originally observed in NRF2 null neurons (Chen et al., 2009; Lee et al., 2003). Taken together, these results show that at least part of DJ-1-mediated neuroprotection likely occurs through the master antioxidant regulator NRF2.

As a previous study had suggested that DJ-1 promotes NRF2 activity through inhibition of its association with its cytosolic inhibitor KEAP1, we tested, and confirmed, the hypothesis that DJ-1 interacts transiently with KEAP1 in an oxidative stress dependent manner. Collectively, The DJ-1-NRF2 axis provides additional insight as to the mechanism by which DJ-1 mediates neuroprotection following cellular insult. In addition, the integration of the previously alluded to DJ-1-AKT link would suggest that DJ-1 confers its NRF2-

mediated antioxidant response through AKT recruitment and activation at the plasma membrane.

The mechanistic work in chapters two and three of this thesis reveals a novel signaling role for DJ-1 in the context of ROS and neuronal death. Specifically, we show that DJ-1 achieves its neuroprotective role through the pro-survival functions of AKT and NRF2. These findings thus shed light onto a new physiological role of DJ-1 in the cell without which may account for PD in humans.

Chapter four of this thesis focused on taking a step back from these cellular and molecular pathways in PD pathogenesis and look into a establishing a better animal model of the disease. To do so, we backcrossed the DJ-1 null mouse that we had previously generated in collaboration with Tak W. Mak (Kim et al., 2005b) to a pure C57Bl6/J background (14 times). These backcrossed mice, termed DJ1-C57, were created in order to perform more comprehensive histological and behavior studies as well as generate double and triple knockout mice of other PD genes.

Interestingly, following extensive interbreeding of these mice, we began to notice selective unilateral degeneration in the SNc of a subset of these DJ-1 null mice (termed DJ1-C57 affected mice). This phenotype was only present in the DJ1-C57 mice and not in their wild-type littermates. In addition, this phenotype appeared to selectively target dopaminergic cells of the SNc and *Locus Ceruleus* while leaving the VTA mostly unaffected: a feature often seen in PD. What's more, this phenotype, originally occurring as early as two months of age, progresses to a more bilateral phenotype with age.

These histopathological findings do not appear to have behavioral correlates. This lack of correlation is likely due to the fact that the losses observed in this model likely mimic

early stage PD since in pre-clinical human PD, individuals with at least 50% of their total SNc dopaminergic neuron pool remaining do not demonstrate clear clinical correlates. Collectively, chapter four presents a novel model of pre-clinical PD that could be used to effectively understand the onset and progression of the early stages of the disease.

Future Directions

The results presented above and others have indicated a role for DJ-1 in handling oxidative stress. Nevertheless, the mechanism(s) through which it accomplishes this function is unclear. This thesis provided a new mechanism through which DJ-1 may signal for the general detoxification of the cell following dopaminergic insult thus providing a potential therapeutic avenue. Nevertheless, broad antioxidants have given inconsistent results with regards to therapeutics in PD and therefore better understanding of these molecular pathways underlying the management of ROS are necessary.

As the functional biochemical relationship between DJ-1 and AKT has yet to be discovered, we can speculate of two principal ways through which DJ-1 may be impacting AKT membrane recruitment. First, DJ-1 may be quenching ROS on a local level within the cell (i.e. the plasma membrane or the mitochondrion) and can thus prevent AKT oxidation and its subsequent inactivation (Durgados et al., 2012). Alternatively, DJ-1 may competitively inhibit, through its chaperone function, other proteins that constitutively inhibit AKT recruitment to the membranous areas of the cell. For instance, recent results obtained from a IP-Mass spectrometry study (collaboration with Daniel Figeys) indicate that DJ-1 may interact with Pleckstrin Homology-Like Domain, family A, member 3 (PHLDA3). This finding is particularly interesting given that PHLDA3 has been shown to be a repressor of AKT through competitive inhibition of its binding to PIP3 (Kawase et al., 2009). Thus, the

hypothesis that DJ-1 may bind and inhibit PHLDA3, specifically under conditions of oxidative stress is an attractive mechanism through which DJ-1 may confer its neuroprotective function.

With regards to NRF2 activation by DJ-1, we hypothesize that DJ-1 acts like an endogenous activator in a similar manner to *t*BHQ. This theory is appealing due to the critical role of the cysteine 106 residue of DJ-1 in its neuroprotective response following oxidative stress (Aleyasin et al., 2007; Blackinton et al., 2009; Canet-Aviles et al., 2004; Junn et al., 2009). Specifically, C106 is thought to become oxidized, where it may accomplish a different function, be it translocation to other compartments of the cell such as mitochondria or plasma membrane, or permit interactions with other gene targets. In the case of NRF2 regulation, we speculate that it is the latter: DJ-1 may permit NRF2 translocation to the nucleus by interacting with KEAP1, through transient disulfide binding, and subsequently permitting NRF2 relinquishment. In this way DJ-1 may act as an endogenous form of classic NRF2 activators such as *t*BHQ. Furthermore, a recent study also demonstrated that *t*BHQ could protect against dopaminergic insult regardless of the DJ-1 genotype thus giving credence to the hypothesis that DJ-1 and *t*BHQ act in a similar manner. This hypothesis is currently being pursued in our laboratory.

From a therapeutic perspective, the concept of DJ-1 functioning as an endogenous form of NRF2 activating drugs, such as *t*BHQ and sulforaphane, is particularly appealing given the high tolerance of the latter compounds. Nevertheless, these findings should be validated further in other animal models of PD before starting exploration of effective DJ-1 activating compounds for potential therapeutic interventions.

Due to the lack of animal models that fully recapitulate PD, the generation of the DJ1-C57 mouse model is of high importance for the field. As the initial characterization of the phenotype is complete, the current effort of our laboratory has two goals. Firstly, we are searching for the causative agent of this phenotype (likely acting synergistically with DJ-1 under physiological conditions). Indeed, our preliminary mouse genealogy studies (autosomal recessive; revealing approximately 1:3 affected: unaffected ratio) would suggest that a single genetic modifier is present in these DJ1-C57 affected mice. In order to address this hypothesis, we have isolated genomic DNA from DJ1-C57 affected and unaffected littermates and subjected it to exome capture (Agilent) followed by next generation sequencing in collaboration with The Center for Applied Genomics (Illumina HiSeq 2000; TCAG, Sick Kids) in order to find an exonic variant that may account for this unilateral phenotype.

If this modifier were identified, we could devise experiments to determine the relationship between it and DJ-1 biochemically and functionally. In addition, one could screen the metadata from previous Genome Wide Association Studies (GWAS) studies to test whether the identified modifier is associated with disease susceptibility. Secondly, we aim to determine whether an observable phenotype may be made by micro Position Emission Tomography (μ PET) scan. As of yet, it is difficult to follow the unilateral phenotype as characterization of this phenotype happens on a histological level. Therefore, effectuating this μ PET study in live animals would help concentrate the phenotype by effectuating DJ1-C57 affected x DJ1-C57 affected crosses and further aid in establishing a mechanism through which these neurons are dying. As a result, this pre-clinical phenotype could be monitored without sacrificing these animals.

This μ PET imaging study is currently being undertaken in collaboration with the PET Imaging Facility at the University of British Columbia (Collaboration with Vesna Sossi). Moreover, this animal model might serve as an effective tool to run diagnostic tests for disease biomarkers that parallel more closely the human condition. Ultimately, understanding how catecholaminergic neurons in the affected DJ1-C57 animals degenerate will likely give us information with regards to the pathogenesis and the progression of this disease.

Collectively, the findings discussed in this thesis bridge cellular, molecular and phenotypic characterization of the gene DJ-1 in an animal model of PD. The addition of these novel findings to the current body of knowledge surrounding genetic and biochemical interaction between DJ-1 and other PD genes as well as various cellular processes are likely to herald future translational approaches. By targeting this disease using this two-tiered method, we garner additional insight into the preexisting mechanisms of physiological neuroprotection surrounding this PD gene while utilizing a novel animal model to provide a platform to study degeneration in a clinically relevant manner.

Appendix I:

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Title: The role of Cdk5-mediated apurinic/apyrimidinic endonuclease 1 phosphorylation in neuronal death

Author: En Huang ,Dianbo Qu ,Yi Zhang ,Katerina Venderova ,M. Emdadul Haque et al.

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Contribution: My contribution to the research was helping in performing part of the in vivo work. I additionally provided help in editing the manuscript following revisions. My overall contribution to this paper was 5%.

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,*}

* These authors contributed equally to this work.

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 peroxidase (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 Cdk5. 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

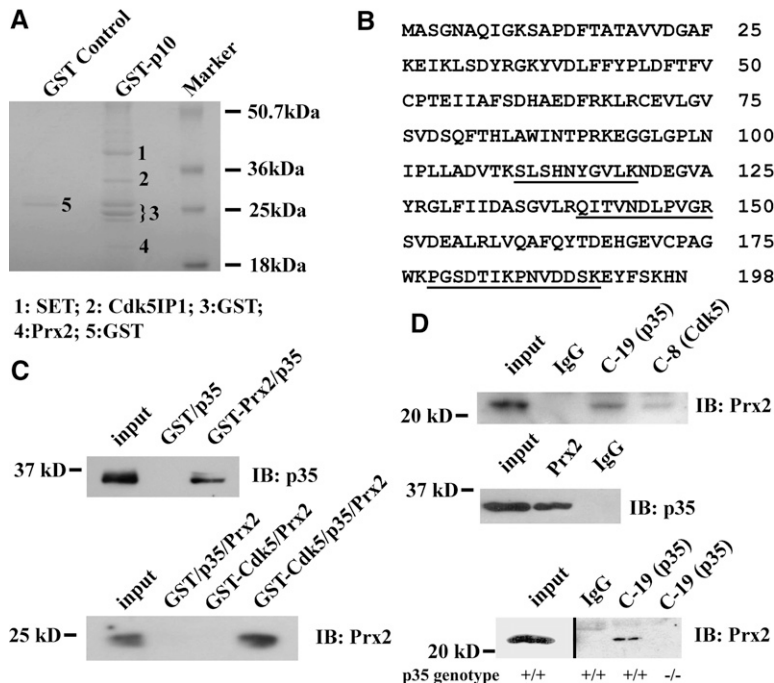


Figure 1. Identification of Prx2 as Cdk5/p35 Interactor

(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, T⁸⁹PRK, optimal for Cdk5 phosphorylation. To investigate whether Prx2 is a substrate of Cdk5, Prx2 and Prx2T89A, a Prx2 mutant in which nonphosphor-

ylatable 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 H₂O₂-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,

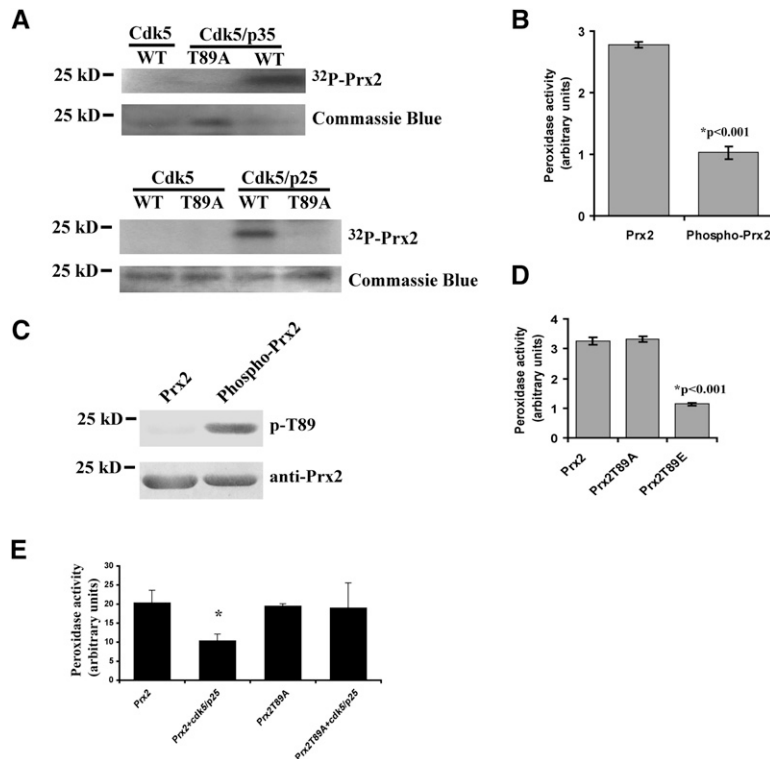


Figure 2. Prx2 Is an In Vitro Substrate of Cdk5, and Peroxidase Activity of Prx2 Is Modulated through Phosphorylation

(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 μ Ci 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 μ g of GST-Cdk5/GST-p25 and 10 μ M 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 μ g 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 $\pm$ 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 μ g of purified His-Prx2, His-

Prx2T89A, or His-PrxT89E was assayed for peroxidase activity as described above in (B). The data is the mean $\pm$ 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 μ g 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 $\pm$ 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 phos-

phorylated 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'-Dichlorofluorescein 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, co-treatment 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

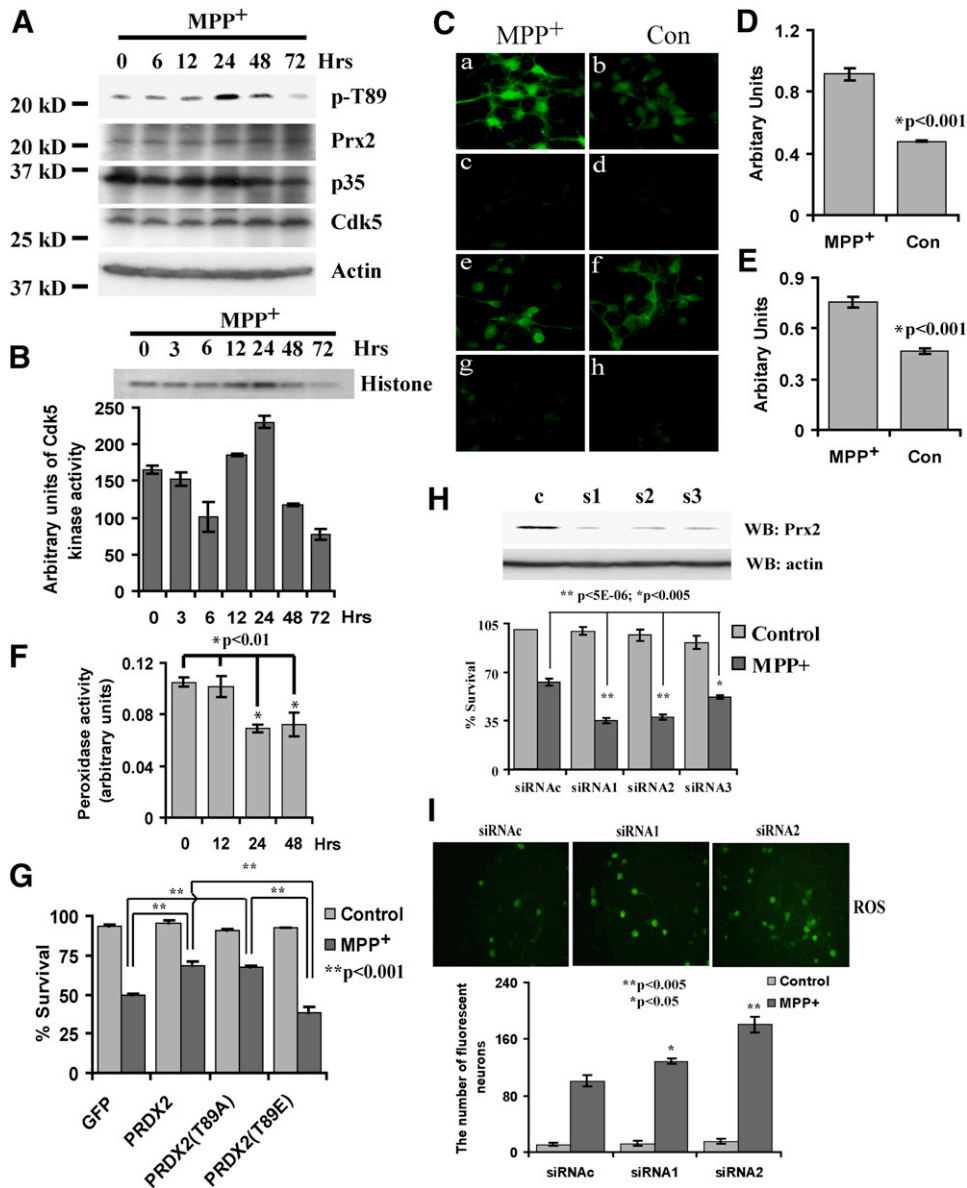


Figure 3. Reduction of Prx2 Peroxidase Activity and Associated Phosphorylation at T89 in Neurons after MPP⁺ Treatment

(A) Increase of phospho-Prx2 after MPP⁺ insult. Cultured cortical neurons were treated with 20 μ M 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 μ g of cell lysate by immunoprecipitation using the C-8 antibody and was incubated with 0.5 μ Ci of [γ -³²P]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 $\pm$ 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 $\pm$ 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 $\pm$ 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 immu-

nofluorescence 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 post-mortem 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 from 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 $\pm$ 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 $\pm$ 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 μ M 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 $\pm$ SEM (n = 4).

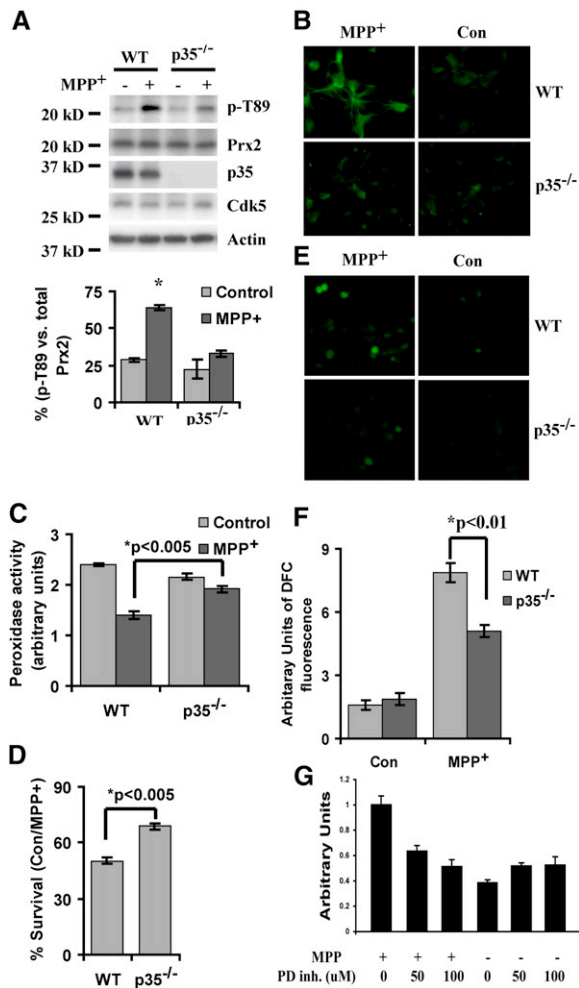


Figure 4. Prx2 Is a Substrate of Cdk5 in Neurons Treated with MPP⁺

(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 re-probed 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 $\pm$ 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 $\pm$ 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 $\pm$ 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 μ M MPP⁺ for 24 hr under a fluorescent microscope. (F) Quantification of DCF fluorescence signal in WT and p35^{-/-} neurons either

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

treated or untreated with MPP⁺. Random fields were analyzed for average fluorescence intensity. The data are the mean $\pm$ SEM (n = 4).

(G) Calpain inhibitors block increase in MPP⁺-induced phospho-Prx2 signal. Cortical neuronal cultures were untreated or treated with 20 μ M 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 $\pm$ SEM.

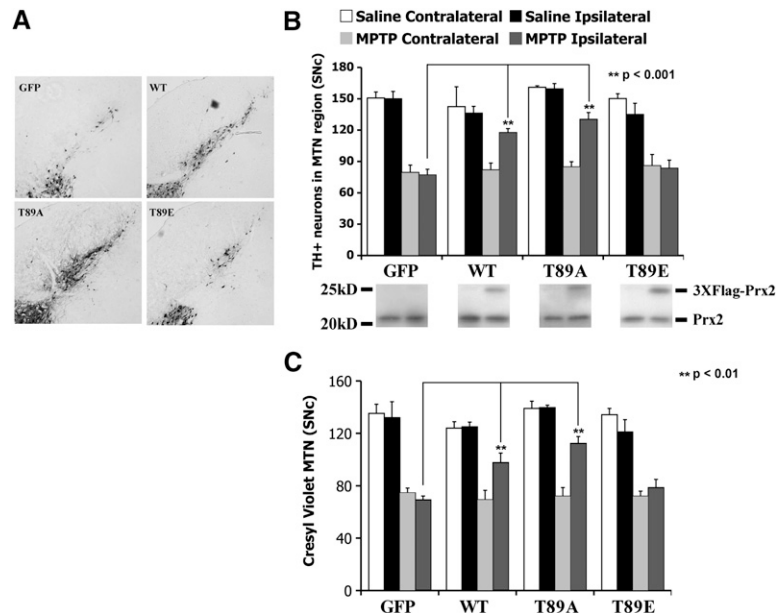


Figure 5. Prx2 Protects DAergic Loss following MPTP Administration

(A) The adenoviruses ($2 \mu\text{l}$, 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 $14 \mu\text{m}$ 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 contralateral for the indicated treatment groups are shown. The data are presented as mean $\pm$ 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 $\pm$ 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 mis-localized 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

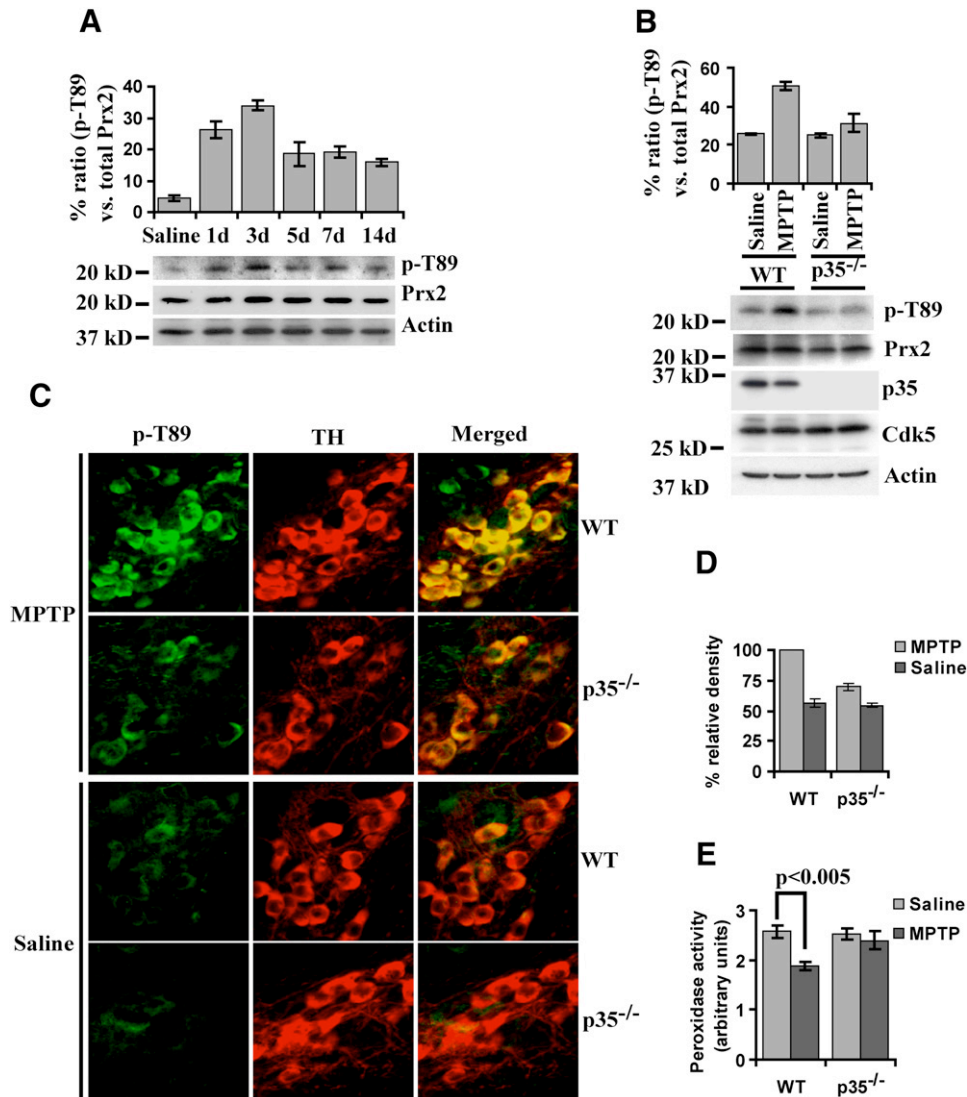


Figure 6. Peroxidase Activity of Prx2 Is Regulated in an In Vivo MPTP Mouse Model of PD

(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 $\pm$ 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 $\pm$ 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 $\pm$ SEM).

(E) Downregulation of peroxidase activity of Prx2 is mediated by Cdk5 in mice administrated by MPTP. Prx2 was isolated from 50 μ g 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 $\pm$ SEM (n = 3).

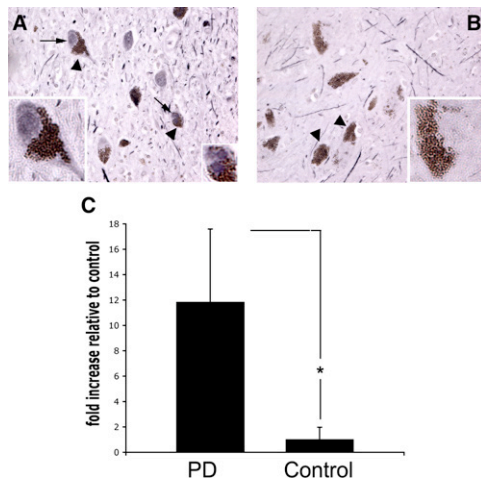


Figure 7. Prx2 Phosphorylation in Human PD

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 $\pm$ 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, par-

ticipates 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

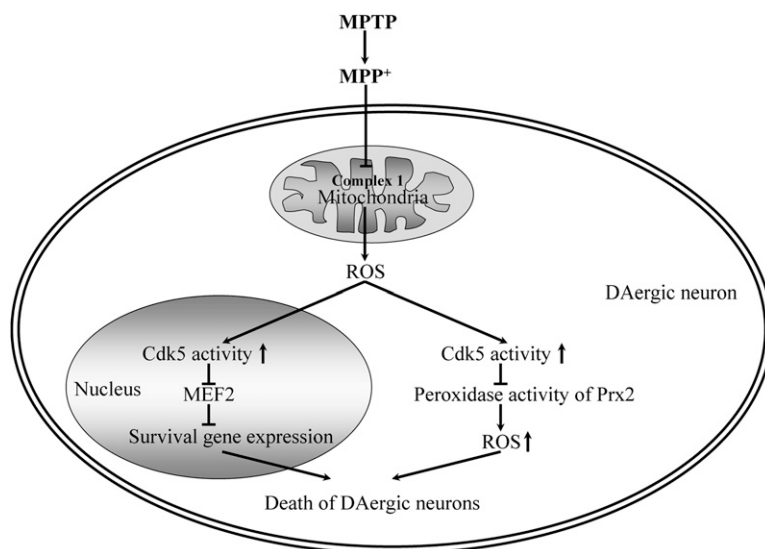


Figure 8. Model of Cdk5-Mediated DAergic Loss in MPTP Mouse Model of PD

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 NcoI/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 μ M 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 μ M 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 μ M 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 μ g of total plasmid DNA (0.75 μ g of plasmid DNA and 0.25 μ g 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 μ M 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 AAUACUAGGCCU) 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 μ M). 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 μ g of bacterially expressed proteins or the precipitated proteins was incubated with 5 μ M Trx, 1 μ M Trx reductase, and 100 μ M NADPH in HEPES (pH 7.5). The reaction was initiated by the addition of H₂O₂ 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 μ l, 1×10^7 particles per μ l) 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 μ l/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 μ m 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 μ g 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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The Parkinson's disease gene DJ-1 is also a key regulator of stroke-induced damage

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Recent evidence has indicated that common mechanisms play roles among multiple neurological diseases. However, the specifics of these pathways are not completely understood. Stroke is caused by the interruption of blood flow to the brain, and cumulative evidence supports the critical role of oxidative stress in the ensuing neuronal death process. DJ-1 (*PARK7*) has been identified as the gene linked to early-onset familial Parkinson's disease. Currently, our work also shows that DJ-1 is central to death in both *in vitro* and *in vivo* models of stroke. Loss of DJ-1 increases the sensitivity to excitotoxicity and ischemia, whereas expression of DJ-1 can reverse this sensitivity and indeed provide further protection. Importantly, DJ-1 expression decreases markers of oxidative stress after stroke insult *in vivo*, suggesting that DJ-1 protects through alleviation of oxidative stress. Consistent with this finding, we demonstrate the essential role of the oxidation-sensitive cysteine-106 residue in the neuroprotective activity of DJ-1 after stroke. Our work provides an important example of how a gene seemingly specific for one disease, in this case Parkinson's disease, also appears to be central in other neuropathological conditions such as stroke. It also highlights the important commonalities among differing neuropathologies.

neurodegeneration | oxidative stress | ischemia | neuroprotection

Stroke is one of the major leading causes of death and disability in North America (1, 2). It is caused by the interruption of the brain blood supply due to occlusion (ischemic) or rupture of blood vessels (hemorrhagic) leading to neuronal dysfunction and death. Although, the complete nature of the complex intra-/extracellular signals that regulate neuronal injury remains to be clarified, a growing body of evidence supports the essential role of oxidative stress in initiation and progression of the injury process (3, 4).

Reactive oxygen species (ROS), free radicals that are normal by-products of oxygen metabolism, are produced in excess during the course of ischemia/reperfusion by a variety of mechanisms such as aberrant electron transport in injured mitochondria (5), calcium influx (6), and inflammatory reactions (7). ROS rapidly react with proteins, lipids, and DNA and cause damage and if severe, cell death. Equally important, they likely activate specific signaling pathways that initiate adaptive or death responses (8). This ability to control ROS is critical in stroke, and neuronal damage occurs if the "oxidant-antioxidant" balance is disturbed in favor of excess oxidative stress during ischemia/reperfusion (9, 10). Therefore, ROS management plays a central role in the pathogenesis of stroke.

DJ-1 (*PARK7*), a protein originally discovered as an oncogene (11), was also identified as an autosomal recessive gene of Parkinson's disease (PD) (12). The physiological role of DJ-1 remains unclear; however, the protective role of DJ-1 against oxidative stress has been shown in several pathological disease models both *in vitro* and *in vivo* (13–15). For instance, previous published data from our laboratory demonstrate the neuropro-

TECTIVE activity of DJ-1 in models of PD induced by the dopaminergic toxins rotenone and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (16). Our results also suggested that DJ-1 may respond to selective initiators of damage, in particular those that involve oxidative stress. This finding was demonstrated by the observation that DJ-1 loss did not sensitize cultured cells to DNA damage- or staurosporine-induced death. These results are consistent with the notion that DJ-1 itself is modified by oxidation (17, 18) and that DJ-1 modifies the transcription of antioxidant enzymes in various cell lines, likely through the regulation of Nrf2, a transcription factor that coordinates a variety of antioxidant enzymes (19). However, whether this result occurs in neurons is unknown.

If the main mechanism of action of DJ-1 is through its ability to handle oxidative stress, then DJ-1 should also be central to other models of neuronal injury where oxidative damage plays a paramount role, including stroke. The question of whether DJ-1 plays this role is of particular interest because there is increasing evidence of the cross-talk between various neurodegenerative diseases. For example, small "silent" strokes are thought to predispose patients to cognitive impairments later in life (20, 21). A significant population of PD patients also suffer from cognitive defects (22). Clearly, mechanisms/genes defined for one neurodegenerative condition might also be central in multiple models of neuropathology. To test these hypotheses, we examined whether the PD gene *DJ-1* may be important in stroke-induced damage. In this work, we demonstrate that DJ-1 deficiency sensitizes brains to ischemic damage *in vivo*, whereas DJ-1 expression does the converse. DJ-1-mediated protection is also accompanied by decreased markers of oxidative damage. Finally, the protective activity depends on cysteine-106 in DJ-1, a residue shown to be critical in the ability of DJ-1 to handle oxidative stress. Our results indicate that a classic PD gene is also central to the ability of the brain to respond to stroke, likely mediated through its capacity to manage ROS.

Results

DJ-1-Null Cerebellar Granule Neurons (CGNs) Are More Sensitive to Excitotoxicity in Model of Death *in Vitro*. To evaluate whether DJ-1 plays any role in stroke-related injury, we first utilized an *in vitro* model of glutamate-induced excitotoxicity. This mechanism of death is critical in stroke. Neuronal death occurs through stimulation of glutamate receptors, subsequent influx of calcium ions, overproduction of ROS, and oxidative damage (23). As is

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The authors declare no conflict of interest.

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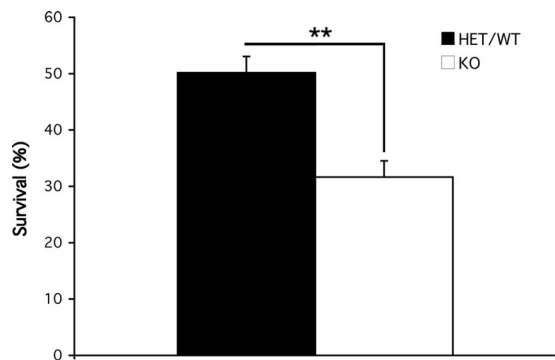


Fig. 1. Increased sensitivity to glutamate-induced excitotoxicity in the absence of DJ-1. CGNs were harvested from DJ-1^{+/-} or ^{+/+} ($n = 7$) and DJ-1^{-/-} ($n = 4$) 7- to 9-day-old mice pups. Seven days after plating, they were treated with 50 μ M L-glutamate for 45 min, washed, and incubated in 37°C for 2 h. Then cells were lysed, and nuclei were fixed and counted on a hemocytometer slide to assess their viability. Each data point is the mean $\pm$ SEM of three independent cultures, **, $P < 0.01$. KO, knockout.

shown in Fig. 1, DJ-1 null neurons are significantly more sensitive to glutamate-induced excitotoxicity than littermate controls [$0.2 \pm 3.8\%$ survival in wild-type (WT) or heterozygous (HET) vs. $31.6 \pm 2.9\%$ survival in DJ-1-deficient animals]. This evidence demonstrates the neuroprotective role of DJ-1 in excitotoxicity, at least *in vitro*, and is consistent with the proposed role of DJ-1 against oxidative stress. Also consistent with this finding, we observed that DJ-1-deficient neurons were also sensitive to hypoxic injury (data not shown).

Oxidation-Sensitive Cysteine-106 Residue in DJ-1 Is Essential for Its Neuroprotective Activities *in Vitro*. We next determined the converse effects of DJ-1 expression on excitotoxic death. As shown in Fig. 2, we expressed GFP alone, GFP with WT DJ-1, or the mutant of DJ-1 (L166P) associated with familial PD. These constructs were targeted to CGNs by recombinant adenoviral vectors (rAV) before glutamate-induced excitotoxicity. Survival was assessed, and it was noted that expression of WT DJ-1 protects from excitotoxic death ($84.8 \pm 0.5\%$ vs. $69.5 \pm 1.3\%$), whereas the PD mutant form of DJ-1 ($66.1 \pm 1.4\%$) proved to display no protective function. Taken together, the evidence indicates that although loss of DJ-1 sensitizes to excitotoxic injury, gain of DJ-1 function is protective.

As was reported previously, DJ-1 responds to oxidative stressors through isoelectric pH (pI) shift to a more acidic form of the molecule (24). Moreover, different independent groups of investigators have demonstrated that the cysteine residue in position 106 is particularly sensitive to oxidative modification and stress (25, 26). To evaluate the importance of this and other cysteine residues in the ability of DJ-1 to protect against excitotoxicity *in vitro*, we also evaluated the ability of C106A or C53A mutant forms of DJ-1 to protect. The latter cysteine mutant is not thought to be modified by as much as cysteine-106 upon oxidative stress (18). As shown in Fig. 2, the mutant C53A form of DJ-1 was still protective compared with control ($87.7 \pm 0.8\%$ vs. $84.8 \pm 0.5\%$), whereas the C106A mutant failed to enhance survival ($73.0 \pm 1.9\%$), indicating the importance of cysteine-106 in mediating protection against excitotoxic death.

DJ-1-Deficient Mice Are Sensitive to Brain Ischemia. Our findings in the *in vitro* models of excitotoxicity provided strong rationale to examine whether DJ-1 is necessary to protect brain tissue against ischemia-induced damage *in vivo*. To examine this hypothesis, we used striatal focal ischemia model by injecting endothelin-1 in the striatum of DJ-1-deficient, heterozygote, and WT control mice.

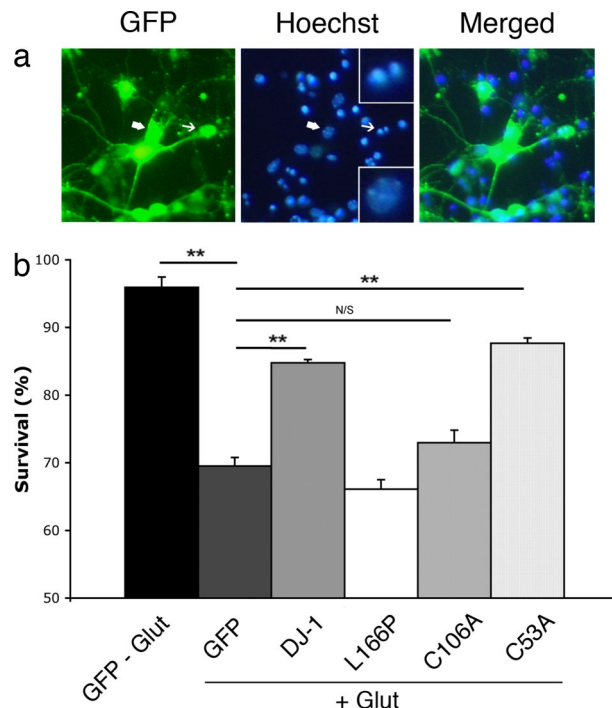


Fig. 2. Cysteine-106 is essential for neuroprotective function of DJ-1 after glutamate-induced excitotoxicity *in vitro*. CGNs, harvested from CD-1 WT pups, were targeted by rAV vectors carrying the indicated constructs along with GFP, 5 days after plating, and treated with L-glutamate at day 7 *in vitro* (as described in *Materials and Methods*). (a) Viability of virally infected neurons was assessed based on nuclear morphology under UV microscopy (representative cells are shown). Condensed or fragmented nuclei (indicated by thin arrows) were scored as dead versus intact nuclei considered as alive (indicated by thick arrows). (Upper Inset) High magnification of fragmented nuclei. (Lower Inset) High magnification of intact nuclei. (b) Quantification of neuronal survival after treatment with L-glutamate compared with non-treated GFP-expressing neurons. Each data point is the mean $\pm$ SEM of three independent cultures (one-way ANOVA followed by Tukey's least significant difference test; **, $P < 0.01$; N/S, not significant).

Endothelin-1 is a vasopressive protein that induces local vasoconstriction around the site of injection, resulting in ischemia-induced tissue damage in affected areas (27). We injected endothelin-1 in two points, 0.4 mm apart in the striatum at coordinates as described in *Materials and Methods*. Animals were killed 7 days after surgery, perfused, and brains were processed as described. The area of damage was determined as presented in Fig. 3b. Endothelin-1 injection in DJ-1-deficient animals produced a significantly larger infarct size (≈ 4 times larger) than either heterozygote or WT control groups. This finding, in line with *in vitro* results, strongly supports the idea of involvement of DJ-1 in neuroprotection in ischemia-induced stroke.

Induction of DJ-1 Can Reverse Ischemia-Induced Damage Sensitivity in DJ-1-Deficient Mice *in Vivo*. Germ-line DJ-1 deficiency might have long-term secondary consequences not directly related to a DJ-1-mediated mechanism of action. To support the importance of DJ-1, we next examined whether induced expression of DJ-1 reverses the sensitization phenotype of DJ-1-null mice in response to endothelin-1-induced ischemic brain damage. We expressed WT DJ-1 in the striatum of DJ-1-null and WT controls by adenoviral vectors a week before initiation of ischemic insult, as described in *Materials and Methods*. Results shown in Fig. 3c indicate that DJ-1 expression reverses the sensitivity to ischemic damage induced by endothelin-1 in DJ-1-null mice and protect them to levels that are not significantly different from WT DJ-1

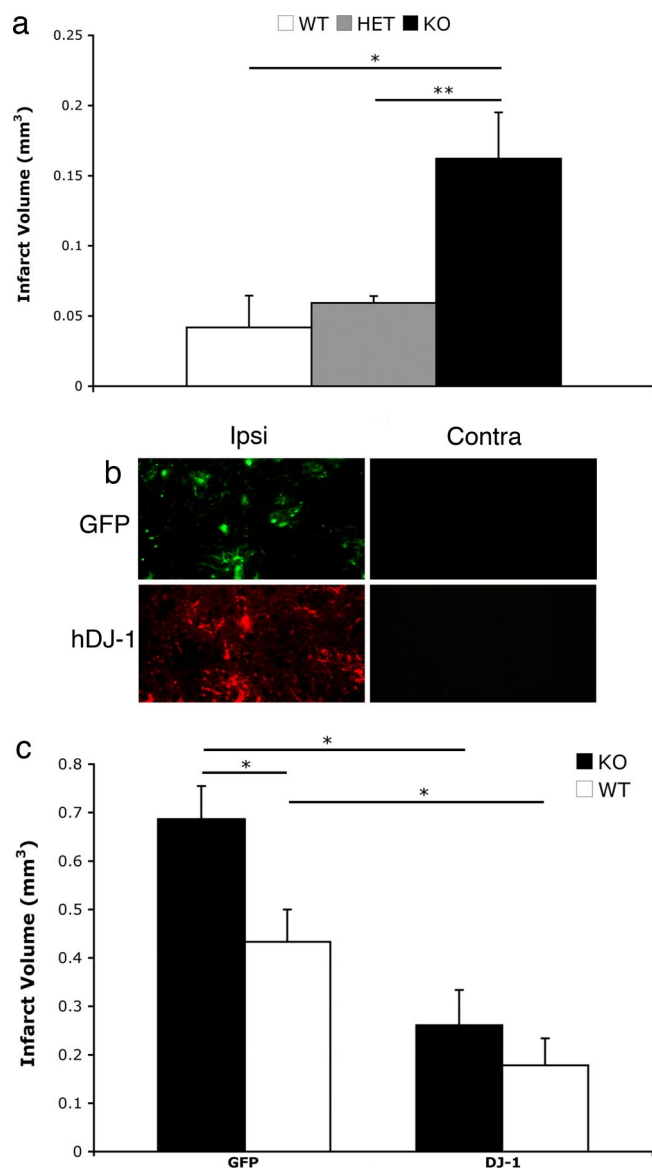


Fig. 3. DJ-1 protects brain tissue from endothelin-induced focal ischemia *in vivo*. Infarct volume in cortex and striatum of mouse brains after acute ischemic stroke is shown. (a) Nonvirally injected mice of DJ-1 colony were assessed for infarct volume after endothelin-induced focal ischemia and were analyzed according to their genotype (WT, $n = 3$; HET, $n = 3$; KO, $n = 4$). There is a significant difference between the animals of the DJ-1-null background vs. either the WT or HET group but no significant difference between the latter two. (b) Expression of either GFP or human DJ-1 was assessed in the ipsi- and contralateral sides of the brains of DJ-1 KO mice (compared with the injected side). (c) Mice of DJ-1-null or WT genotype from the DJ-1 colony were injected with adenoviral vectors expressing either GFP (KO, $n = 5$; WT, $n = 7$) or DJ-1 (KO, $n = 6$; WT, $n = 8$). Animals in each group received the same previously mentioned stroke model procedure. Seven days later, brain infarct volume was measured as described. There were significant differences between DJ-1-null + GFP-injected and DJ-1-null + DJ-1-injected as well as an additional protection seen in the WT overexpressing DJ-1 as opposed to overexpressing GFP. No significant changes were observed between the WT overexpressing GFP and the KO overexpressing DJ-1. Each data point represents mean $\pm$ SEM for a representative population of three to eight mice (one-way ANOVA followed by Tukey's least significant difference test; *, $P < 0.05$; **, $P < 0.01$).

mice. Furthermore, it was observed that DJ-1 overexpression in the control group provided additional protection against ischemic injury. It must be noted that the degree of sensitization induced by DJ-1 deficiency in control GFP-expressing mice is

less than that observed in naïve nonvirally injected animals (Fig. 3). This effect may be caused by the response of animals to viral infection. Nonetheless, taken together, these findings strongly support the role of DJ-1 in protecting neuronal cells against the ischemic damage model of stroke.

Cysteine-106 Is Essential for the Protective Role of DJ-1 in Ischemic Brain Injury. Our *in vitro* experiments indicated the requirement of the oxidation-sensitive cysteine-106 in the neuroprotective activity of DJ-1 in excitotoxicity. To evaluate the significance of this residue in the protective function of DJ-1 in our adult stroke models of injury *in vivo*, we evaluated the effects of DJ-1, WT and mutants, in our model of endothelin-induced damage. In this case, we used the rat model, which, based on our previous experience, produces larger infarct volumes in response to endothelin-1. This model facilitates the precise evaluation of the protective effects of DJ-1. In addition, we also used the adeno-associated viral (AAV) vectors to express wild-type DJ-1, C106A and C53A mutants, or GFP control in rat brains. AAV is known to be an effective, neurotrophic, and reliable method of gene delivery that produces a stronger and broader gene expression than with adenovirus (28). We injected AAV vectors 2 weeks before endothelin-1 injection to allow sufficient expression of the proteins of interest (Fig. 4b). One week after the endothelin-1 injection, we transcardially perfused the rats and prepared the brains for cryostat sectioning. Brain serial sections were examined to evaluate the volume of infarct (Fig. 4a). As demonstrated in Fig. 4c, the volume of infarct is significantly reduced in WT DJ-1- and C53A mutant-expressing brains compared with GFP-expressing brains (1.79 ± 0.36 and 1.91 ± 0.33 vs. 5.86 ± 0.55 , respectively). Importantly, however, the protective effect of C106A was as greatly compromised compared with WT DJ-1 (4.20 ± 0.45 vs. 1.79 ± 0.36 , respectively). These results clearly demonstrate that cysteine-106 is essential for protective function of DJ-1 in this model of ischemic stroke.

DJ-1-Mediated Protection Is Associated with Reduced Markers of Oxidative Stress. Ischemia/reperfusion is believed to provoke neuronal damage by increasing production of ROS. To evaluate the level of ROS after ischemic insult, we used 8-oxoguanine (8-oxoG), an oxidized by-product of purine base of DNA, as an indicator of oxidative damage (29). We performed a preliminary time course and observed that 8-oxoG reactivity in tissue reaches its maximum at 48 h after ischemia (data not shown). If DJ-1 acts to promote survival through its antioxidant properties, we would expect that ROS level and consequent oxidative damage after stroke would decrease in the presence of WT or neuroprotective mutants of DJ-1. To test this possibility, we assessed the level of 8-oxoG reactivity by immunohistochemistry staining and comparing signal intensity between injected vs. noninjected hemispheres in the presence of overexpressed GFP, WT, or mutant forms of DJ-1, 48 h after ischemia. Densitometry analysis presented in Fig. 5 clearly indicates that WT DJ-1 as well as C53A mutant effectively reduce poststroke ROS (0.92 ± 0.12 and 1.07 ± 0.04 , respectively) compared with the measurements in C106A and GFP, which are comparable (2.20 ± 0.12 vs. 1.90 ± 0.24 , respectively).

Discussion

There is increasing evidence that common mechanistic elements may play central roles in a variety of neurodegenerative and neuropathological conditions. Chief among these elements is ROS. Free radical damage has been implicated in arguably every neuropathological condition described. The importance of ROS is underscored by the elegant system of enzymes developed by the cell to manage and balance ROS under normal conditions and times of stress. These antioxidant enzymes include catalases,

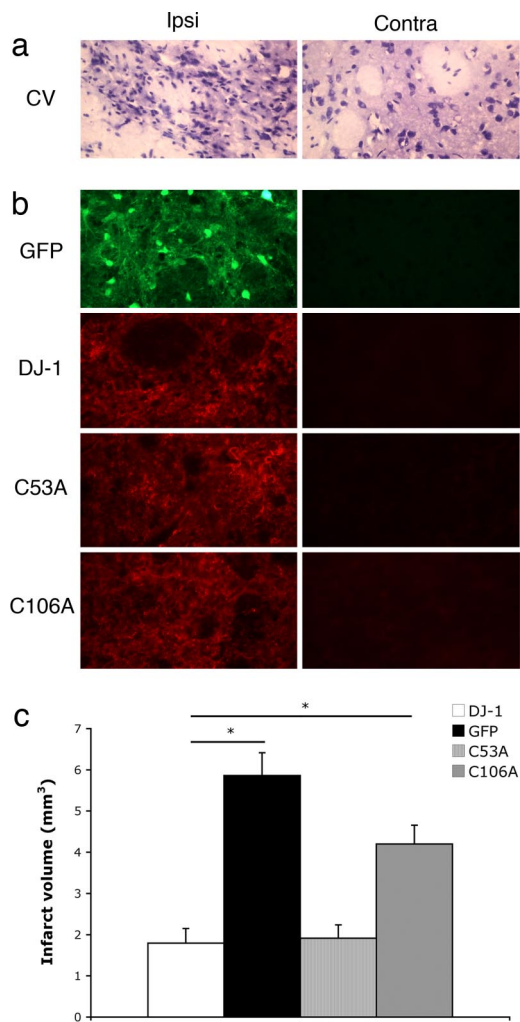


Fig. 4. Cysteine-106 is essential for neuroprotective function of DJ-1 after focal ischemia *in vivo*. Infarct volume in the striatum after injection of endothelin-1 in WT rats overexpressing AAVs was assessed. (a) Representative sections of damaged (ipsilateral to the endothelin-1 injection) and healthy (contralateral) cells were stained with cresyl violet, and damaged areas were characterized through shrunken/condensed nuclear staining (dead cell) vs. healthy neurons with smooth, round nuclei and visible nucleoli (alive). (b) Sections from the injected hemisphere overlapping the infarcted area were stained for respective virally expressing protein. (c) Infarct volume was measured by cresyl violet staining between different virally injected groups ($n = 4$ for GFP and DJ-1; $n = 3$ for C53A and C106A). Each data point represents mean $\pm$ SEM for a representative population of rats. There were significant differences between DJ-1 and GFP overexpressing rats as well as C53A and GFP but not C106A and GFP overexpressing rats (one-way ANOVA followed by Tukey's least significant difference test; *, significant). No significant changes were observed between DJ-1 and C53A overexpressing rats.

glutathione peroxidases, thioredoxin reductases, and superoxide dismutases (30).

A more recently identified protein linked to ROS is DJ-1. DJ-1 was first identified by many groups in the context of fertility and oncogenesis (11, 31). However, more recent excitement was generated with its identification as a familial PD gene (12). The reason why loss of DJ-1 leads to PD is still unknown. However, growing evidence suggested a link to oxidative stress (17, 24, 32). For example, we had previously shown that DJ-1 deficiency protects against direct peroxide-induced death but not death initiated by DNA damage or staurosporine (16). The possible mechanisms for its link to oxidative stress will be discussed in

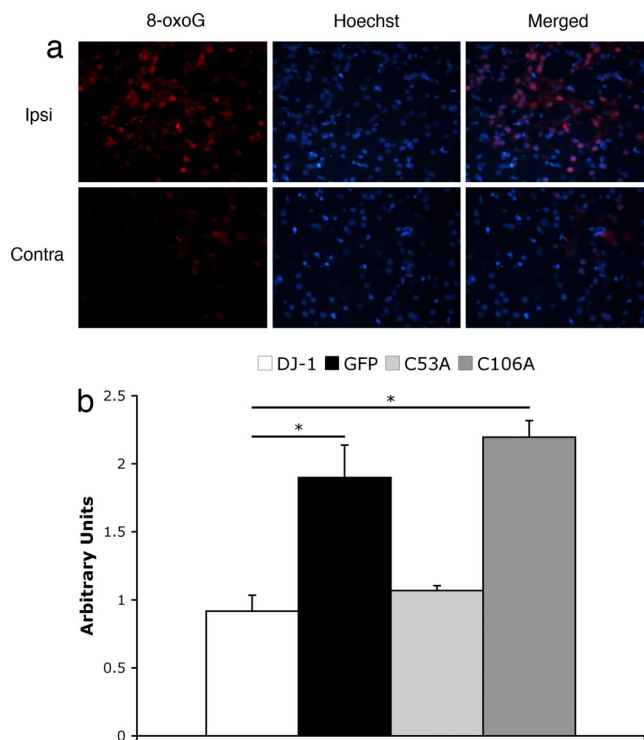


Fig. 5. Cysteine-106 is crucial for the regulation of ROS end product, 8-oxoG after focal ischemia *in vivo*. Rats subjected to AAV injection overexpressing DJ-1, GFP, C53A, and C106A, as indicated, were subjected to ischemic insult by endothelin-1 and were killed after 48 h. Brains were collected and processed to be stained for 8-oxoG as well as Hoechst 33258. Representative pictures were taken of sections surrounding injection area, and colocalizing 8-oxoG/Hoechst neurons ($n > 50$) were evaluated by densitometry compared with the contralateral side. (a) Representative sections show expression of ROS marker 8-oxoG on the ipsi- and contralateral sides of the endothelin injection. (b) Quantification of densitometry results. Arbitrary units represent the ratio of signal density for the injected vs. noninjected (internal control) sides. Each data point represents mean $\pm$ SEM for a representative population of three or four rats per group. There is a significant difference between DJ-1 and GFP overexpressing groups as well as DJ-1 and C106A overexpressing rats (one-way ANOVA; followed by Tukey's least significant difference test; *, significant). No significant changes were observed between DJ-1 and C53A groups.

more detail below. However, if the link between DJ-1 and oxidative stress was valid, one logical prediction would be that DJ-1 should play important roles in other non-PD-related neuropathological conditions also associated with ROS. In this regard, stroke-induced damage is an obvious test candidate because ROS is strongly induced after ischemic damage, and amelioration of ROS has been shown to prevent ischemic injury (33). Accordingly, in the present work, we demonstrate the critical impact of DJ-1 in *in vitro* and *in vivo* models of ischemic stroke. Our results indicate that (i) DJ-1 provides protection against excitotoxicity and ischemic brain injury and (ii) antioxidant activity of DJ-1 is essential to its neuroprotective role against ischemia-induced neuronal damage.

We initially examined the role of DJ-1 in glutamate-induced excitotoxicity as a critical pathogenic event during stroke (34). Our data show that DJ-1-null CGNs are significantly more sensitive to glutamate treatment than the controls harvested from littermate pups. Consistent with this observation, overexpression of human DJ-1 in mouse CGNs considerably protects them against glutamate-induced death. This sensitivity also extends to adult models of stroke injury *in vivo* where we observed that DJ-1-deficient mice displayed larger infarct volumes than control littermates. More importantly, this sensitivity

was reversible through induction of human DJ-1. The importance of DJ-1 in modifying survival is also underscored by the observation that expression of DJ-1 alone is protective against ischemic stress, both *in vitro* and *in vivo*. Importantly, expression of human DJ-1 provides additional protection, even in DJ-1 WT mice and rats. Our results are significant because they show how a gene classically associated with one neurological disease, in this case PD, also plays pivotal roles in other conditions such as stroke. Our results are consistent with increasing evidence of commonalities among neuropathologies. These commonalities extend not only to signaling elements including those classically associated with death such as JNKs, p53, but broader mechanistic elements such as mitochondrial damage, endoplasmic reticulum stress, and of course free radical damage to name just a few (35–38). In further support of this view, there appears to be overlap between etiologies and even pathologies of a number of diseases. For example, strokes predispose patients to dementia (21). PD patients have symptoms not classically associated with movement such as dementia and mood disorders. Accordingly, in the context of the present work, it would be interesting to determine whether PD patients with DJ-1 deficiency are also more sensitive to other neuropathologies such as stroke.

There are several lines of evidence to support the role of DJ-1 in handling ROS in stroke. First, expression of DJ-1 diminishes markers of oxidative stress induced by stroke insult. In this regard, our results show that the 8-oxoG epitope, which represents that the oxidized form of guanosine, is increased after stroke and reduced after DJ-1 expression (Fig. 5). Second, we also show that mutants of DJ-1 with known defects in responsiveness to oxidative stress do not protect against stroke. In this regard, the C106A mutant of DJ-1 failed to protect against ischemic damage, both *in vitro* and *in vivo*. The cysteine-106 residue was initially reported to be the most sensitive amino acid residue to hydrogen peroxide oxidation (18). Subsequent studies have shown that this residue to also critical for the protective properties of DJ-1 in response to oxidative stress (25). Our results are consistent with this observation and give further support to the importance of DJ-1 and its ability to handle oxidative stress in stroke.

The exact mechanism by which DJ-1 and the cysteine-106 residue protect neurons from ROS stress is unclear. Direct quenching and *in vitro* catalase activity of DJ-1 has been reported previously (39). Whether this activity is central to diseases such as stroke is unknown. DJ-1 is also suggested to enhance prosurvival signals such as AKT (39, 40). However, its relevance in neurodegeneration again is unclear. Recent data also indicate that DJ-1 can stabilize the transcription factor, Nrf2 (19). This finding is particularly interesting because Nrf2 is known to coordinate the expression of a number of antioxidant genes. Interestingly, we have shown that DJ-1 can modify activation of an important antioxidant enzyme Prdx2 (41). Whether and how these mechanisms impact on DJ-1, PD, or stroke will be of intense interest. Taken together, our findings in this work provide direct evidence to support the neuroprotective role of DJ-1 in mammalian animal model of brain ischemic injury. Given the central nature of ROS in stroke, DJ-1 may be an important new target in the prevention of stroke damage.

Materials and Methods

Cell Culture. Primary cultures of CGNs were obtained from postnatal day 7–9 CD1 or DJ-1 null colony mouse pups, as described previously (42).

Viral Constructs. Recombinant AAV (rAAV1) vectors were constructed by subcloning cDNA sequences (NheI–PmeI fragment) of WT human DJ-1 or mutated forms (C53A and C106A) into the SpeI–EcoRV sites of the AM/CBA-pl-WPRE-bGH plasmid. The virus was then generated and purified as described (43). For

adenovirus (AV) construction, the same sequences were subcloned into the pAdTrack vector under a cytomegalovirus (CMV) promoter. The construct also contains a second CMV promoter that separately controls expression of GFP. The construct was then used to generate recombinant AV, as described (44).

Adenoviral Infection and Glutamate Excitotoxicity. Five days after plating, CGNs were infected with adenovirus-expressing GFP along with DJ-1 WT or DJ-1 mutants (L166P, C53A, C106A) or by GFP itself as control (multiplicity of infection, 20). On day 7, L-glutamate was added to the wells to a final concentration of 50 μ M for 45 min and then washed three times with conditioned medium and incubated for 2 h in 37°C. This step was performed in the presence or absence of 10 μ M MK801. All cultures were fixed in 4% paraformaldehyde [containing 0.2% picric acid in 0.1 M phosphate buffer (pH 6.9)], and their nuclei were stained with Hoechst 33258 (final concentration of 0.5 μ g/ml). The total number of GFP-positive neurons that had healthy nuclei (round intact nuclei vs. shrunken condensed or fragmented nuclei) per well was evaluated and compared with the number of GFP-positive live neurons in control wells.

DJ-1-Null Mice, Colony Maintenance, and Genotyping. DJ-1-deficient mice were generated by deletion of exons 3–5 and replacement with a NeoTD cassette, maintained, and genotyped by PCR as described previously by Kim *et al.* (16). Animals were maintained at 25°C on a 12-h/12-h light/dark cycle with access to standard rodent laboratory chow and water. All animal procedures were in the accordance with the guidelines of the Canadian Council and Care of Animals in Research and the Canadian Institutes of Health Research and were approved by the University of Ottawa Animal Care Veterinary Services.

Intrastriatal Viral Injection and Focal Ischemia Induction. Surgical procedures were performed as described by Rashidian *et al.* (45). Briefly, mice of the DJ-1 colony (C57BL/6 background 12 generations, 8–12 weeks old) or male Wistar rats weighing 75–100 g (Charles River) were anesthetized with a mixture of 2–2.5% isoflurane and 1 liter/min oxygen, and after proper sterilization and wellness procedures, they were placed in a stereotaxic frame. Stereotaxic intrastriatal AV (1 μ l, 1×10^7 particles per injection, infusion rate, 0.25 μ l/min) or rAAV injection [3 μ l, premixed with 30% (vol/vol) mannitol, 1.2×10^9 particles per injection, infusion rate, 0.15 μ l/min] for mice (left side, from bregma: +0.5 mm anteroposterior, +1.9 mm lateral, –2.9 mm deep) and rats (left side, from bregma: +0.9 mm anteroposterior, +2.8 mm lateral, –5.8 mm deep) were performed 7 and 14 days before ischemic insult, respectively. To induce ischemic insult in rats, we injected 1 μ l of vasoconstrictor, endothelin-1 (1 mg/ml dissolved in ddH₂O; Calbiochem) into the same virally injected striatal region (infusion rate of 0.125 μ l/min). For mice, the endothelin-1 injection was repeated twice, in two points of the left striatum distanced 0.4 mm apart (from bregma: +0.5 mm anteroposterior, +2.1 and +1.7 mm lateral, –2.9 mm deep) for a total volume of 2 μ l.

Histological Staining and Infarct Volume Assessment. Animals were anesthetized and transcardially perfused 7 days after endothelin-1 injection with 0.9% saline followed by 4% paraformaldehyde (PFA). Brains were then removed, fixed overnight in 4% PFA, and dehydrated over a course of 5 days in 10% (wt/vol) sucrose in 0.1 M phosphate buffer. Coronal sections of 14 μ m were obtained by using a Microm HM500 cryomicrotome. Serial sections were taken starting at bregma +1.54 mm and ended at bregma –0.94 mm to include the whole striatal area. One of five consecutive sections of the striatum was collected, refixed in 4% PFA, and stained with 0.2% cresyl violet. The damaged tissue surface area, determined on

nuclear morphology (areas with condensed/shrunken nuclei were considered infarcted), was outlined by using Northern Eclipse imaging software (Empix Imaging), and infarct volume was assessed by multiplying the area of damage by the thickness of section as well as the gap between selected slices. All microscopic and imaging studies were performed by using a Zeiss microscope (Axiophot).

Immunohistochemistry. Forty-eight hours after endothelin-1 administration, animals in the indicated groups were perfused, and brains were fixed as described before. Free-floating coronal cryosections of 14 μm were obtained (bregma +2.70 mm and ended at bregma -2.12 mm). Sections were then blocked in 2% normal goat serum with 1% BSA and incubated with 8-oxoG (1:300; Chemicon) monoclonal anti-mouse antibody overnight at 4°C. After three washes with PBS, tissue was stained with

secondary CY3-conjugated secondary antibody (1:300; Jackson ImmunoResearch) for 1 h at room temperature. Slides were washed, and coverslips were mounted with GelMount Aqueous Mounting Medium (Sigma).

Statistical Analysis. All statistical variance was carried out by using Student's *t* test (two-tailed) or one-way ANOVA followed by Tukey's post hoc least significant difference test. Significance: *, $P < 0.05$; **, $P < 0.01$ unless otherwise stated.

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Contribution: My contribution to the research consisted of performing *in vivo* stroke work in addition to analyzing and producing figures for publication. I further contributed in the editing of the manuscript for submission and revisions. My overall contribution to this paper was 15%.

Essential Role of Cytoplasmic cdk5 and Prx2 in Multiple Ischemic Injury Models, *In Vivo*

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Recent evidence suggests that abnormal activation of cyclin-dependent kinase 5 (cdk5) is a critical prodeath signal in stroke. However, the mechanism(s) by which cdk5 promotes death is unclear. Complicating the role of cdk5 are the observations that cdk5 can exist in multiple cellular regions and possess both prosurvival and prodeath characteristics. In particular, the critical role of cytoplasmic or nuclear cdk5 in neuronal injury, *in vivo*, is unclear. Therefore, we determined where cdk5 was activated in models of ischemia and how manipulation of cdk5 in differing compartments may affect neuronal death. Here, we show a critical function for cytoplasmic cdk5 in both focal and global models of stroke, *in vivo*. Cdk5 is activated in the cytoplasm and expression of DNcdk5 localized to the cytoplasm is protective. Importantly, we also demonstrate the antioxidant enzyme Prx2 (peroxiredoxin 2) as a critical cytoplasmic target of cdk5. In contrast, the role of cdk5 in the nucleus is context-dependent. Following focal ischemia, nuclear cdk5 is activated and functionally relevant while there is no evidence for such activation following global ischemia. Importantly, myocyte enhancer factor 2D (MEF2D), a previously described nuclear target of cdk5 *in vitro*, is also phosphorylated by cdk5 following focal ischemia. In addition, MEF2D expression in this paradigm ameliorates death. Together, our results address the critical issue of cdk5 activity compartmentalization, as well as define critical substrates for both cytoplasmic and nuclear cdk5 activity in adult models of stroke.

Introduction

Stroke results from a transient or permanent reduction in blood flow to the brain. The mechanisms involved in ischemic neuronal death are not fully defined. In this regard, accumulating evidence has implicated cyclin-dependent kinases (CDKs) with stroke damage (Rashidian et al., 2007). CDKs are best recognized for their role in regulating cell cycle progression (Pines, 1993). However, they are now noted for their roles in other biological processes (Gold and Rice, 1998). Specifically, some specialized members are predominately active in the nervous system. Cdk5, a proline-directed serine/threonine kinase, is one such member. Cdk5 does not use cyclins to promote activation. Instead, it uses p35 and p39 which are predominantly expressed in CNS (Dhavan and Tsai, 2001). Moreover, in contrast to other CDKs, there is little functional evidence for involvement of cdk5 in classical cell cycle regulation. Instead, cdk5 is considered a key element in neuronal development and function (Dhavan and Tsai, 2001; Li et al., 2001; Johansson et al., 2005). Cdk5 is also believed to be

critical for neuronal survival following exposure to apoptotic stimuli (Li et al., 2002, 2003; Wang et al., 2006).

Interestingly, in contrast to its normal function, there is increasing evidence that suggests a pathogenic role of cdk5 in neurodegenerative disorders, such as Parkinson's disease (PD) (Smith et al., 2003), Alzheimer's disease (AD) (Cruz and Tsai, 2004), amyotrophic lateral sclerosis (ALS) (Nguyen and Julien, 2003), Niemann-Pick type C (NPC) (Bu et al., 2002), and stroke (Wang et al., 2003; Rashidian et al., 2005). This pathogenic face is proposed to be unmasked by calpain-mediated cleavage of the p35 to a more stable active p25 form. There is evidence of increasing levels of p25 in the nucleus, which could alter cdk5 substrate specificity under some neurotoxic conditions (Patrick et al., 1999; Kusakawa et al., 2000; O'Hare et al., 2005). p35 contains a myristoylation motif and associates with membranous compartments of the cell, while p25 lacks this motif and localizes cdk5 to soluble fractions (Kusakawa et al., 2000). Moreover, several studies have demonstrated that cdk5 can be both nuclear and cytoplasmic (Ino and Chiba, 1996; O'Hare et al., 2005; Fu et al., 2006). Recent evidence suggests that cell localization might be important for its prosurvival/prodeath role. We have recently shown that inhibition of cytoplasmic cdk5 sensitizes neurons to DNA damage, suggesting a prosurvival role of cytoplasmic cdk5 under normal conditions (O'Hare et al., 2005). In contrast, nuclear cdk5, in an *in vitro* model of excitotoxicity is death promoting, through inhibition of the survival properties of myocyte enhancer factor 2D (MEF2D) (O'Hare et al., 2005; Tang et al., 2005). This would suggest

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cytoplasmic activity is normal while nuclear activity is pathogenic. However, this simplistic view is likely incorrect. For example, cdk5 can phosphorylate tau in the cytoplasm, a presumptive prodeath stress (Cruz and Tsai, 2004). These observations suggest that cdk5 activity is much more complex. Moreover, the role of cdk5 localization in adult models of injury, particularly in stroke is unclear. Understanding of where cdk5 is activated and contributes to pathogenesis is critical, particularly in light of its prosurvival function(s).

Presently, we determine the localization of cdk5 activity in multiple models of stroke *in vivo* and provide critical downstream targets for such activity. We provide evidence that cytoplasmic cdk5 activity is essential for death in multiple paradigms of ischemia (focal and global) *in vivo*, while nuclear cdk5 activity is only activated following focal ischemia. Critically, we also identify peroxiredoxin 2 (Prx2) as a common critical cytoplasmic target and the transcription factor MEF2D as a relevant nuclear target in stroke. These observations highlight the complexity of cdk5 activation and define at least two critical cdk5 signaling axes in stroke.

Materials and Methods

Animals. All animal experiments were approved by the University of Ottawa Animal Care Committee and conformed to the guidelines set forth by the Animal Care Council of Canada and Canadian Institutes of Health Research.

Viral construction. Recombinant adeno-associated virus (AAV) vectors were constructed by subcloning cDNA sequences of DNcdk5-Flag (Gong et al., 2003; Smith et al., 2003), Wtcdk5-GFP-NLS(NES), DNcdk5-GFP-NLS(NES) (O'Hare et al., 2005), and Prx2-Flag and its mutants (Prx2T89A, Prx2T89E) in the AM/CBA-pl-WPRE-bGH plasmid. Then, viruses were generated as previously described (Zolotukhin et al., 2002). The adenoviruses (AV) expressing Prx2, Prx2T89A, Prx2T89E, and MEF2D-S444 were engineered by subcloning these sequences into pAdTrack vectors under control of a CMV promoter. These vectors also expressed green fluorescent protein (GFP) by a separate CMV promoter. Finally, these constructs were used to generate AV, as described previously (He et al., 1998).

P35 knock-out mice. P35^{-/-} mice were generated by breeding heterozygote p35 mutants and genotyped as described previously (Hallows et al., 2003).

Cell culture. Primary cerebellar granule neuron (CGN) cultures were prepared from 7–9 d postnatal mice as previously described (Rashidian et al., 2005).

Glutamate model of neuronal death. Five day plated CGN cultures in 96-well plates were infected with AV with multiplicity of infection of 20 (MOI = 20) for survival assay. On day 7, cultures were exposed to 50 μ M glutamate for 60–70 min, in the presence or absence of MK801, and then washed with conditional medium and incubated for 90 min. Cultures were then fixed with 4% paraformaldehyde (PFA) and stained with Hoechst 33342 (0.25 μ g/ml; Sigma-Aldrich) for nuclei staining. Nuclei from live cells appeared intact and noncondensed or nonfragmented while nuclei from dead cells appeared condensed or fragmented. For the survival assay, the numbers of live GFP-positive cells were assessed in each entire well. Three wells for each treatment were counted and averaged. To calculate percentage of live CGNs after glutamate treatment, we compared the number of live GFP-positive neurons obtained in the absence of MK801 with the number of live GFP-positive neurons obtained in the presence of MK801 for each virus. MK801 is an NMDA receptor blocker, which inhibits >90% of death induced by glutamate treatment in this paradigm. We used the MK801/glutamate-treated cultures as control for any minor nonspecific death induced by media change. Survival is expressed as percentage of live CGNs as described by (number of live GFP-positive neurons in the absence of MK801/number of live GFP-positive neurons in the presence of MK801) $\times$ 100.

For detecting phosphorylation of WtPrx2 and Prx2T89A, 5 d plated CGN cultures in 4-well plates were infected with AV (MOI = 20). On

day 7, cultures were exposed to 20 μ M glutamate for 20 min and then washed with conditional medium and incubated. Cultures were collected for Western blot analyses 0, 30, 60 and 90 min following incubation.

Viral injection. Male Wistar rats weighing 80–100 g were unilaterally injected with AAV vectors carrying DNcdk5-Flag, Wtcdk5-GFP-NLS(NES), DNcdk5-GFP-NLS(NES), WtPrx2-Flag, Prx2T89A-Flag, Prx2T89E-Flag, or GFP as control 2 weeks before induction of ischemia. AAV was diluted by mixing 2 μ l of virus stock (10^{10} genomes/ μ l) with 1 μ l of 20% mannitol and administered into the hippocampus (from bregma: -3.6 mm anteroposterior, $+2.1$ mm lateral, -2.75 mm deep; in 4VO model) or striatum (from bregma: $+0.9$ mm anteroposterior, $+2.8$ mm lateral, -5.8 mm deep; in endothelin model) using an infusion pump (Harvard Infusion Pump) over a 30 min period (0.1 μ l per minute) as described previously (Rashidian et al., 2005). AV carrying MEF2D-S444, a cdk5 phosphorylation mutant of MEF2D or GFP was delivered into striatum, using above coordinates. Unilateral injection of virus was given to each animal (3 μ l; 3×10^7 particles/ μ l) one week before injection of endothelin-1 as described previously (Smith et al., 2003).

Global ischemia induced by 4-vessel occlusion. Five minute 4-vessel occlusion (4VO) was induced in male Wistar rats weighing 180–220 g. One day before global ischemia insult, the common carotid arteries were exposed by a ventral midline neck incision and looped with suture. Both vertebral arteries were permanently occluded by electrocauterization. The animals were allowed to recover for 24 h. The next day, the carotid arteries were exposed and the rats were allowed to recover from anesthesia till they reacted to a tail-pinching stimulus. At this time, both common carotid arteries were quickly occluded for 5 min using clips. During the occlusion, body temperature was monitored with a rectal probe and maintained at 37°C. After removing clips, rats were kept in a 37°C incubator for 1 h and then moved to individual cages. Only rats that were unresponsive during the procedure and following reperfusion were included in the study. Brains were collected and stained for hematoxylin and eosin (H&E) 4 d after surgery. Cell counts from the left and right hippocampi were averaged and expressed as counts/mm for CA1 region.

Focal ischemia induced by injection of endothelin-1. Endothelin-1 was injected into the striatum of the male Wistar rats (from bregma: $+0.9$ mm anteroposterior, $+2.9$ mm lateral, -6 mm deep) weighing 180–220 g or 8–12 weeks old male mice (from bregma: $+0.5$ mm anteroposterior, $+2.1$ mm lateral, -2.9 mm deep). Endothelin-1 was dissolved in sterile distilled water to a final concentration of 400 pM/ μ l. One microliter (in rats) or 2 $\times$ 1 μ l (in mice; double injection, 0.4 mm apart) of the endothelin-1 solution was injected over a period of 3 min into the striatum using an infusion pump. Brains were collected 4 d after injection and 14 μ m-coronal sections of striatum were stained with cresyl violet (CV). The infarct volume was measured using the method described previously (Swanson et al., 1990). Briefly, for every fifth section, the area of damage was marginated and quantified using image analyses (Northern Eclipse). Infarct volume was calculated by multiplying the area of damage by number of sections and thickness of sections (14 μ m).

Immunohistochemistry. Fourteen micrometer coronal sections at the level of striatum were collected as freefloating in 0.01 M PBS + 0.02% sodium azide. Then sections were permeabilized and blocked in buffer A (50 mM Tris, pH 7.5 + 100 mM NaCl + 0.3% Triton X-100) plus 3% BSA overnight. Next, sections were washed in buffer A and incubated in anti-pT89Prdx2 antibody (Qu et al., 2007) coincubated with a blocking peptide for specificity at 4°C overnight. Then slides were visualized using Alexa Fluor 488 anti-rabbit IgG (1:2000; Invitrogen).

Western blot analyses. To detect expression of DNcdk5, Wtcdk5-GFP-NLS(NES) and DNcdk5-GFP-NLS(NES), Prx2 and its variants, MEF2D and pS444MEF2D in brain, a 2 mm punch was obtained and analyzed by Western blot using anti-Flag (1:4000; Sigma-Aldrich) or anti-cdk5 (1:1000; Santa Cruz Biotechnology), anti-GFP (1:5000; Abcam), anti-Prdx2 (1:500; Abcam), anti-pT89Prx2 (1:500; Qu et al., 2007), anti-MEF2D (1:2000; BD Transduction Laboratories), and anti-pS444MEF2D (1:500) (Gong et al., 2003) antibodies, respectively. Histone H1 (1:1000; Santa Cruz Biotechnology) and Raf-1 (1:1000; Santa Cruz Biotechnology) were

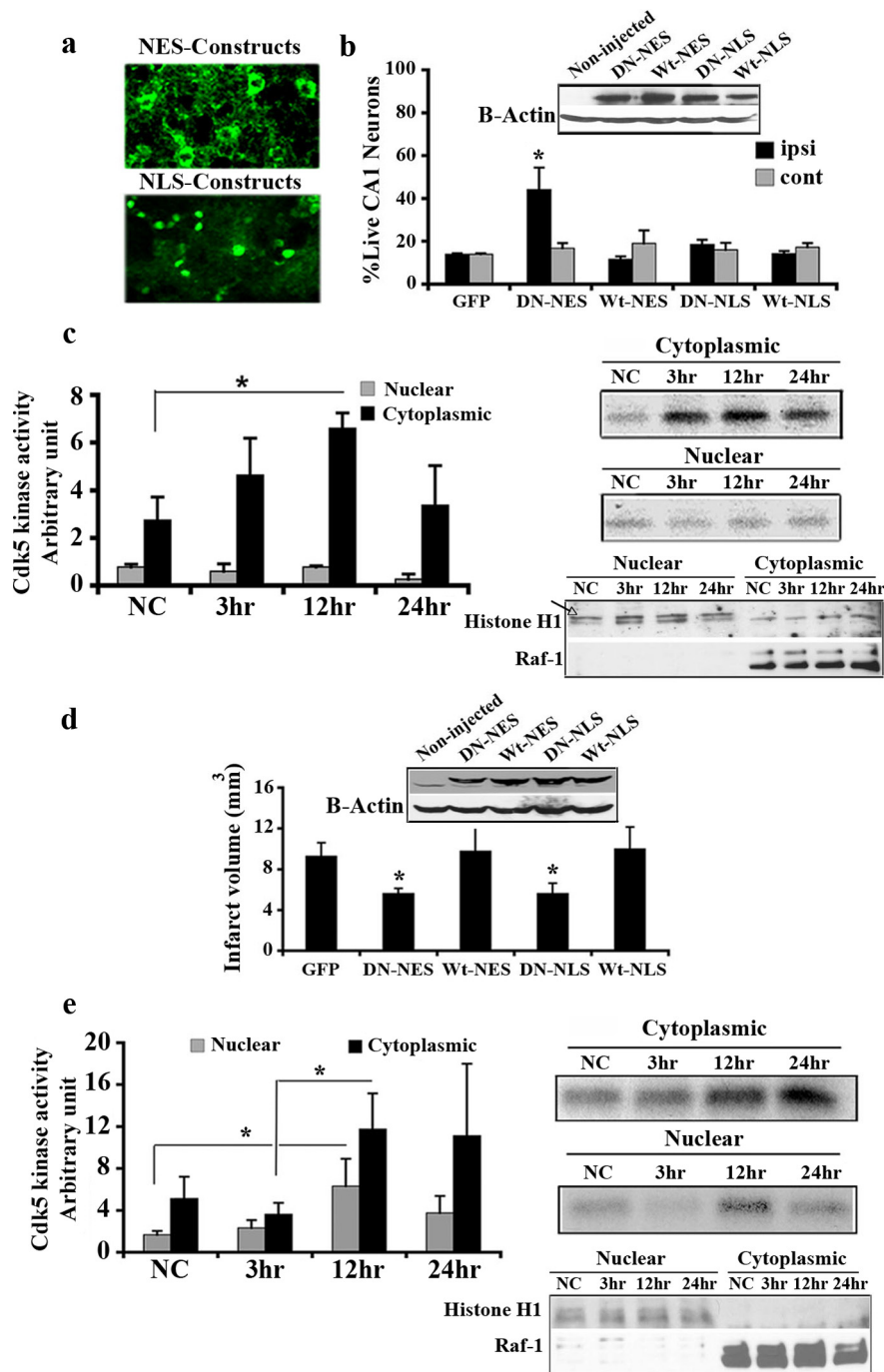


Figure 1. Cytoplasmic cdk5 is mediator of neuronal death following global ischemia, while in focal ischemia both nuclear and cytoplasmic cdk5 signal for death. *a*, AAV-mediated expression of Wt(DN)cdk5-NLS in the nuclei and Wt(DN)cdk5-NES in the cytoplasm was detected using GFP fluorescence in the striatum. *b*, Quantification of surviving CA1 neurons expressing GFP ($n = 5$), DNcdk5-NES ($n = 5$), Wtcdk5-NES ($n = 5$), DNcdk5-NLS ($n = 5$), and Wtcdk5-NLS ($n = 5$) 4 d after 4VO. Expression of constructs in CA1 was shown by Western blot analysis. *c*, Cdk5 kinase assay on nuclear and cytoplasmic proteins from hippocampus using histone H1 as substrate at the different time points after 4VO. The graph presents densitometry values from $n = 4$ experiments. *d*, Infarct volume of focal ischemic brains expressing GFP ($n = 7$), DNcdk5-NES ($n = 7$), Wtcdk5-NES ($n = 8$), DNcdk5-NLS ($n = 7$), and Wtcdk5-NLS ($n = 7$) 4 d after injection of endothelin-1. Expression of constructs in striatum was shown by Western blot analysis. *e*, Cdk5 kinase assay on nuclear and cytoplasmic proteins from striatum using histone H1 as substrate at the different time points after injection of endothelin-1. The graph presents densitometry values from $n = 5$ experiments. Fractionation was assessed using histone H1 as nuclear marker and Raf-1 as cytoplasmic marker in both *c* and *e*. "NC" represents nonstroked control animals; ipsi is injected and cont is noninjected side. Data are presented as mean $\pm$ SEM (*Student's *t*-test, $p < 0.05$).

used as nuclear and cytoplasmic markers, respectively. To detect viral expressed Prx2 variants in CGNs, 2-d-infected cultures were collected and subjected to Western blot using anti-Flag antibody. β -Actin (1:3000; Sigma-Aldrich) was used as loading control for all Western blots.

Cdk5 kinase assay. Cdk5 kinase assay was performed on hippocampal CA1 or striatal tissue samples. One-hundred micrograms of protein was extracted from samples and incubated overnight with anti-cdk5 antibody (2 μ g/sample; Santa Cruz Biotechnology). Thirty microliters of 50% slurry of Protein A-Sepharose (Sigma-Aldrich) was then added to immunoprecipitates, and samples were incubated for 2 h at 4°C. The immune complexes were next pelleted and washed in PBS and incubated with 2 μ g of histone H1 (Boehringer) and 1 μ Ci [32 P] ATP at 30°C for 30 min. Kinase activity was determined by SDS-PAGE and autoradiography.

Extraction of nuclear and cytoplasmic proteins. Hippocampal CA1 or striatal proteins were obtained using a 2 mm punch and nuclear and cytoplasmic proteins were extracted as described before (Wang et al., 2002). Western blot analyses for histone H1, as a nuclear marker, and Raf-1, as a cytoplasmic marker, were performed to evaluate purity of fractions.

Results

Differential roles of cytoplasmic and nuclear cdk5 in multiple models of stroke

We have previously provided evidence that cdk5 plays an essential role in a focal model of stroke induced by exposure to the vasoconstrictor endothelin-1. It is also functionally relevant in a 5 min global model of ischemia (Rashidian et al., 2005) (see also supplemental Material 1, available at www.jneurosci.org), but not following longer duration (10 min) of 4VO. In the latter case, cdk4 appears to play a more central role (Rashidian et al., 2005). Importantly, the mechanism(s) by which cdk5 might promote ischemic death is unknown. As the first step in deciphering the cdk5 localization promoting death *in vivo*, we examined whether nuclear or cytoplasmic cdk5 was essential for death in two models of global and focal ischemia. To determine this, we constructed and used recombinant AAV expressing a wild-type (Wt) or kinase-dead dominant-negative (DN) mutant of cdk5. DNcdk5 carries a point mutation within its catalytic site and is inactive. Presumably, it can competitively inhibit endogenous cdk5 activity by binding to its activators or its substrates. Wtcdk5 and DNcdk5 were fused to GFP (for visualization) as well as either a nuclear exclusion signal (NES) for expression exclusively in the cytoplasm [implicated as Wt(DN)cdk5-NES] or a nuclear localization signal (NLS) for targeting to the nucleus [implicated as Wt(DN)cdk5-NLS]. We have shown previously that these fusion constructs target to either cytoplasm or nucleus (O'Hare et al., 2005). We also presently show by GFP fluorescence imaging that this pattern of exclusive expression in the nucleus or cytoplasm

plasm also occurs in the brain *in vivo* (Fig. 1a). For the global model of ischemia, recombinant AAV vectors carrying these constructs or GFP, as control, were injected unilaterally into hippocampus 2 weeks before induction of 5 min 4VO. Expression of the constructs was confirmed by Western blot analyses (Fig. 1b). Survival of CA1 neurons was assessed 4 d after 4VO by staining of coronal sections of the brain with H&E. Live CA1 neurons are characterized by round soma and intact nuclei, while dead neurons appear shrunken with pyknotic nuclei (supplemental Material 2, available at www.jneurosci.org). Neuronal counts for the CA1 region showed significant increase in survival in the DNcdk5-NES-injected side compared with the noninjected side (44% survival vs 16%) (Fig. 1b). In comparison, overexpression of all other constructs, including DNcdk5-NLS, did not result in any significant survival (Fig. 1b). These results suggest that activation of cytoplasmic, but not nuclear, cdk5 leads to neuronal death following 4VO. To confirm that cytoplasmic cdk5 activation may be more critical in transmitting the death signal, we assessed cdk5 kinase activity in both the cytoplasm and nucleus after 4VO. Cdk5 was

immunoprecipitated from nuclear and cytoplasmic extracts of CA1 at different time points following 4VO. Fractionation was confirmed using histone H1 as nuclear marker and Raf-1 as cytoplasmic marker (Fig. 1c). The samples were then subjected to *in vitro* kinase assay using histone H1 as substrate. As Figure 1c indicates, cytoplasmic cdk5 activity dramatically increased 3 h following reperfusion, with a maximum level at 12 h (2.5 folds). In contrast, cdk5 activity did not increase in the nucleus. This suggests that abnormal activation of cytoplasmic cdk5 has an essential role in neuronal death following 4VO.

The above results were quite surprising to us given previous results from our laboratory (O'Hare et al., 2005) as well as others (Gong et al., 2003), that, at least in *in vitro* cultured neurons, nuclear cdk5 is critical for excitotoxic death. This suggests that the role of cdk5 *in vivo* in the adult animals might differ from that of immature neurons grown in the dish. To examine this possibility, we also performed similar protection experiments as described above in another adult model, a focal ischemia model induced by endothelin-1. Recombinant AAV vectors carrying the DNcdk5 or Wtcdk5 constructs described above or GFP, as control, were injected into the striatum 2 weeks before injection of endothelin-1. Expression of constructs was confirmed by Western blot analyses (Fig. 1d). Regions of striatum with infarct were distinguished by CV staining 4 d after injection, as described previously (Rashidian et al., 2005). In contrast to the 4VO model, there was a very significant decrease (40%) in damage in both DNcdk5-NLS and DNcdk5-NES expressing brains, compared with GFP-expressing brains (Fig. 1d). Overexpression of Wtcdk5 in cytoplasm or nucleus, however, was not protective (Fig. 1d). This indicates that the participation of nuclear and cytoplasmic cdk5 differs in various models of stroke. In a focal model, both nuclear and cytoplasmic cdk5 signal for death in excitotoxic type of death. We also assessed cdk5 kinase activity in the cytoplasm

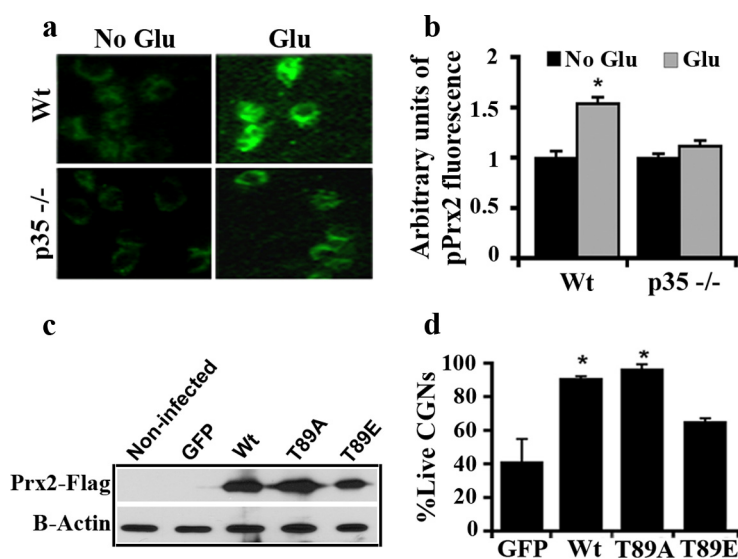


Figure 2. Prx2 is phosphorylated at Thr89 by cdk5 in glutamate model and causes neuronal death. *a*, Phosphorylation of Prx2 is attenuated in p35-deficient CGNs compared with cultures from Wt littermates following glutamate (Glu) excitotoxicity. Cultures were fixed with 4% PFA after glutamate exposure and immunostained for pT89Prx2. *b*, Quantification of surviving CGNs after glutamate exposure. CGNs from each mouse (Wt $n = 7$; p35^{-/-} $n = 4$) were plated on 3 wells and intensity of fluorescent signal in soma of neurons was measured by image analyses from 60 neurons in 4 random fields/well. *c*, AV-mediated expression of WtPrx2, Prx2T89A, Prx2T89E in CGNs was shown by Western blot analysis using anti-Flag antibody. *d*, Overexpression of Prx2 protects CGNs against glutamate. Five day plated CGNs were infected with AV-expressing Wt, phosphorylation resistant (T89A), and phospho-mimicking (T89E) mutants of Prx2 or only GFP as control and exposed to glutamate 2 d after infection. Fixed cultures were then counted for live GFP expressing neurons. Graph is representative of $n = 3$ experiments. Data are presented as mean $\pm$ SEM (*Student's *t*-test, $p < 0.05$).

and nucleus after injection of endothelin-1 similar to that performed after global insult. As Figure 1e indicates, cdk5 activity increased 12 h after injection, compared with negative controls in both cytoplasm and nucleus. This activation is slightly slower than that observed following 4VO. However, this delay can be explained by the fact that in the focal ischemia model induced by endothelin, occlusion and reperfusion are likely much more gradual than with global ischemia.

Together, these results suggest the central nature of cytoplasmic cdk5 as a key mediator of ischemic death in multiple models of ischemia, *in vivo*. However, the activity of nuclear cdk5 does not always participate in the death process.

Prx2 is a substrate for cytoplasmic cdk5 in ischemic death

We have recently reported that Prx2, a cytoplasmic peroxidase, is a cdk5 target (Qu et al., 2007). This antioxidant enzyme is phosphorylated at Thr89 and inactivated by cdk5. Therefore, we examined whether Prx2 may act as a downstream target for cytoplasmic cdk5 in our models of ischemia.

In vitro model of ischemia

We first examined the importance of Prx2 in *in vitro* model of death induced by glutamate in CGNs. CGNs provide a relatively homogenous population of neurons and are efficiently killed by stroke-related insults, such as glutamate treatment. We have previously reported that cdk5 is essential for excitotoxic neuronal death induced by glutamate in CGNs (Rashidian et al., 2005). We first evaluated the effect of glutamate on Thr89 phosphorylation using the phospho-specific antibody pT89 in CGN cultures from Wt and p35^{-/-} mice. The increase in pT89Prx2 was observed in cultures by immunofluorescence and was quantified over a number of neurons by image analyses (Fig. 2a). Quantification indicated a maximum of 50% increase in pT89Prx2 signal in

glutamate-treated Wt CGNs compared with nontreated controls. In contrast, p35^{-/-} cultures showed only 10% statistically non-significant increase upon glutamate treatment, compared with controls (Fig. 2*b*). These results indicate that Prx2 is phosphorylated by cdk5 upon glutamate exposure. To determine whether this phosphorylation is relevant in this paradigm, we infected CGNs with AV expressing WtPrx2, a mutant of Prx2, which cannot be phosphorylated at the Thr89 (Prx2T89A), or a phosphomimicking form, which we have shown to be less active (Prx2T89E), before glutamate treatment. Expression of these constructs was confirmed by Western blot analyses (Fig. 2*c*). The wells treated with MK801/glutamate served as controls. This was to control for nonspecific death caused by washing (which typically accounts for <10% of cells; supplemental Material 3, available at www.jneurosci.org). As shown in Figure 2*d*, neurons exposed to glutamate expressing WtPrx2 and Prx2T89A showed ~90% survival versus 40% survival in GFP-expressing controls. Expression of Prx2T89E showed dramatically less protection than the other Prx2 constructs. Interestingly, Western blot analysis revealed that at least some of the virally expressed WtPrx2 is phosphorylated on Thr89 following glutamate treatment, consistent with observations from endogenous Prx2 (supplemental Material 4, available at www.jneurosci.org). The resistant form Prx2T89A, on the other hand, did not show increase in phosphorylation status (data not shown). Together, this data suggests that phosphorylation of Prx2 on Thr89 by cdk5 in the glutamate model triggers death pathways in CGN cultures.

In vivo models of ischemia

To confirm the above results *in vivo*, we next examined whether Prx2 may be important in the global model of ischemia. We generated AAV expressing WtPrx2, Prx2T89A, and Prx2T89E, and targeted these constructs to the hippocampus before 4VO. Expression of these constructs was confirmed by Western blot analysis (Fig. 3*a*). Evaluation of CA1 neurons showed that expression of WtPrx2 and Prx2T89A significantly protected this region from ischemia (38% and 28% live, respectively) compared with expression of Prx2T89E (15% live) or GFP (14% live) (Fig. 3*a*). The above evidence indicates that Prx2 may be important for ischemic damage. To show a connection between Prx2 and cdk5, we determined whether Prx2 is phosphorylated on Thr89 following ischemia in a cdk5-dependent manner. We first analyzed for total Prx2 and pT89Prx2 by Western blot at different time points following insult. As Figure 3*b* shows, both total Prx2 and pT89Prx2 levels were increased at 3 h, with maximum levels at 12 h after reperfusion. Moreover, the specific activity of phosphorylation (pT89Prx2 to total Prx2) also showed 2.8-fold increase compared with control animals. To ensure that this phosphorylation is cdk5-dependent, DNcdk5 was expressed unilaterally in hippocampus before 4VO. As demonstrated in Figure 3*c*, expression of DNcdk5 attenuated Prx2 phosphorylation. Together, the above data suggests that Prx2 is a functionally relevant cytoplasmic cdk5 target following global ischemia.

Similar results with Prx2 were also observed in a focal model of ischemia. We virally overexpressed WtPrx2, mutants Prx2T89A and Prx2T89E, or GFP in striatum before injection of endothelin-1. Expression of these constructs was confirmed by Western blot analysis (Fig. 4*a*). As Figure 4*a* shows, expression of Prx2T89A, the phosphorylation resistant mutant, provided significant protection from ischemia (58% smaller infarct compared with expression of GFP alone). In contrast, expression of Prx2T89E, the phospho-mimicking mutant, did not offer any protection. Interestingly, unlike in the global model, we did not see sig-

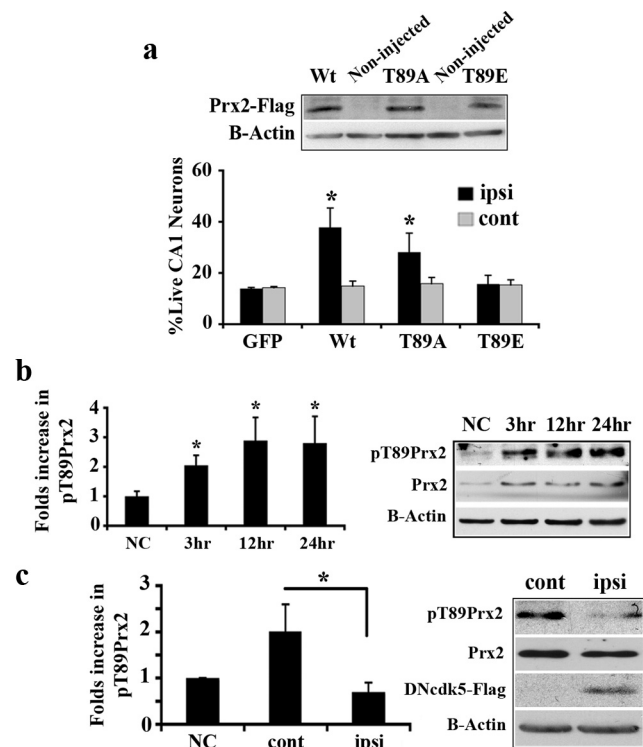


Figure 3. Prx2 is phosphorylated by cdk5 in global ischemia and leads to neuronal death. *a*, Quantification of surviving CA1 neurons expressing GFP ($n = 5$), Wt ($n = 11$), as well as T89A ($n = 10$) or T89E ($n = 6$) forms of Prx2 after 4VO. AAV-mediated expression of WtPrx2, Prx2T89A, Prx2T89E in hippocampus was detected by Western blot analysis using anti-Flag antibody. *b*, Analysis of pPrx2 level in hippocampus at the time points after 4VO by Western blot. The membrane is representative of $n = 6$ experiments and graph presents densitometry values of pPrx2 relative to Prx2. *c*, Inhibition of cdk5 attenuates pPrx2. Rats were unilaterally injected with AAV expressing DNcdk5 into hippocampus and subjected to 4VO. Total proteins from both sides of the hippocampus were analyzed for pPrx2 by Western blot at 24 h after 4VO. Expression of DNcdk5 has been shown using anti-Flag antibody. The membrane is representative of $n = 3$ experiments and graph presents densitometry values of pPrx2 relative to Prx2. ipsi, Virus-injected side; cont, noninjected side. "NC" represents nonstressed control animals. Data are presented as mean $\pm$ SEM (*Student's *t*-test, $p < 0.05$).

nificant protection with WtPrx2. As it has been shown in supplemental Material 5, available at www.jneurosci.org, viral expressed WtPrx2 is phosphorylated at Thr89 24 h after injection of endothelin-1. The reason why WtPrx2 protects following global but not focal insult is unknown but may have to do with timing/degree of phosphorylation of the exogenously expressed Prx2. Alternatively, other death signals might play a role in the focal, but not global model, including signaling in the nucleus (see below).

To determine whether endogenous Prx2 is phosphorylated in this focal model, striatal protein samples were analyzed for total Prx2 and pT89Prx2 by Western blot. As Figure 4*b* shows, both total Prx2 and pT89Prx2 levels were increased. Similar to the global model, the levels of pT89Prx2 relative to total Prx2 were elevated 2.7 fold 24 h after stroke. To test whether phosphorylation of Prx2 was cdk5-mediated, we examined the effects of cdk5 inhibition in two ways. First, we expressed DNcdk5 and measured its effect on Prx2 phosphorylation. As shown in Figure 4*c*, such expression attenuated phosphorylation induced by endothelin-1. Second, we also examined the effects of p35 deficiency. We first measured the extent of damage induced by endothelin-1. As Figure 4*d* demonstrates, p35^{-/-} mice displayed significantly smaller

infarct (50%) compared with wild-type mice. Finally, we performed immunofluorescence analysis for phospho-Prx2 on the coronal sections obtained at the level of striatum. In $p35^{-/-}$ sections, the increase in pT89Prx2 signal observed upon ischemia was significantly attenuated compared with the ones obtained from wild type (Fig. 4e). Thus, p35 deficiency effectively prevents Prx2 phosphorylation and reduces damage following stroke insult. Together, the above data suggests that Prx2 is a critical cytoplasmic cdk5 target, which is phosphorylated and inactivated following focal ischemic injury.

MEF2 is a substrate for nuclear cdk5 in ischemic death

Our above results identify the cytoplasmic cdk5-Prx2 pathway as a critical signaling axis of ischemic neuronal death. In addition, our data demonstrates that under selective conditions, nuclear cdk5 also appears to play a role. To further examine the mechanisms by which this nuclear cdk5 activity promotes neuronal death, we investigated the survival promoting transcription factor MEF2D. Previous evidence indicates that MEF2D is a direct target for cdk5-mediated neurotoxicity, at least in cultured neurons. Cdk5 has been reported to phosphorylate MEF2D on Ser444, which leads to its inactivation (Gong et al., 2003). Accordingly, we next determined whether this nuclear target plays an important role in excitotoxicity induced by endothelin-1. We first performed Western blot analysis on protein samples extracted from endothelin-1-injected striatum using a specific anti-pS444MEF2D antibody. As shown in Figure 5a, MEF2D is phosphorylated at Ser444 following ischemia with the maximum levels at 24 h. In addition, the level of total MEF2D is decreased in a time-dependent manner (Fig. 5a). This is in agreement with previous studies, which showed that phosphorylation of MEF2D by cdk5 promotes its degradation (Tang et al., 2005). In contrast to focal ischemia, neither the levels of pS444MEF2D, nor total MEF2D change following global ischemia (Fig. 5a). This is consistent with the notion that nuclear cdk5 activity is not induced with the latter insult. To confirm that the phosphorylation observed following focal insult depends on cdk5 activity, we examined the effect of cdk5 inhibition by expression of DNcdk5. As Figure 5b shows, expression of DNcdk5 attenuated pS444MEF2D. To further decipher the role of MEF2D phosphorylation in our model, we virally expressed a mutant form of MEF2D, which cannot be phosphorylated at Ser444 (MEF2D-444), or GFP into the striatum of rats, which were subsequently subjected to focal ischemia. As shown in Figure 5d, the phosphorylation-resistant MEF2D significantly protected against damage and resulted in a smaller infarct (66% smaller) compared with GFP-injected control brains.

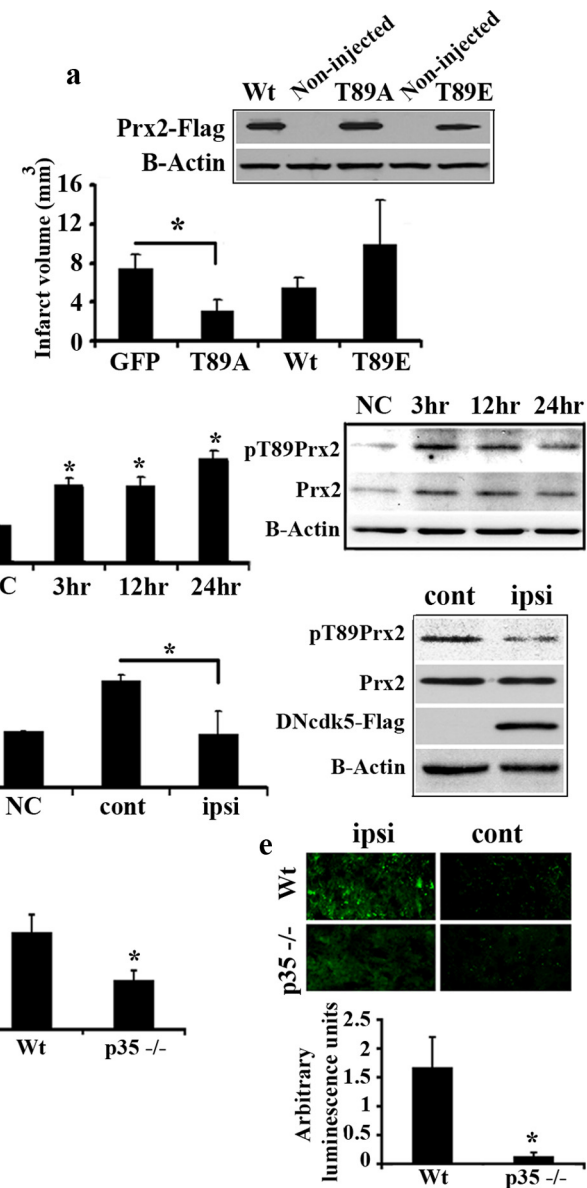


Figure 4. Prx2 is phosphorylated by cdk5 in focal ischemia and causes neuronal death. *a*, Quantification of infarct volume in striatum overexpressing GFP ($n = 5$), Wt ($n = 7$), as well as T89A ($n = 5$) or T89E ($n = 4$) forms of Prx2 following injection of endothelin-1. AAV-mediated expression of constructs in striatum was detected by Western blot analysis using anti-Flag antibody. *b*, Analysis of pPrx2 level in striatum at the time points following endothelin-1 injection by Western blot. The membrane is representative of $n = 3$ experiments and graph presents densitometry values of pPrx2 relative to Prx2. *c*, Inhibition of cdk5 attenuates pPrx2. Rats were unilaterally injected with AAV expressing DNcdk5 and then endothelin-1 into both sides of striatum. Total proteins from both sides of the striatum were analyzed for pPrx2 by Western blot at 24 h after ischemia. Expression of DNcdk5 has been shown using anti-Flag antibody. The membrane is representative of $n = 3$ experiments and graph presents densitometry values of pPrx2 to Prx2. ipsi, Virus injected side; cont, noninjected side. "NC" represents nonstoked control animals. *d*, p35-deficient mice are resistant to focal ischemia. p35^{-/-} ($n = 5$) or Wt ($n = 7$) mice were injected with endothelin-1 in striatum and the infarct volume was measured. *e*, Increased pPrx2 signal is attenuated in p35 deficient mice in focal ischemia. Sections from mice brains (Wt $n = 5$; p35^{-/-} $n = 3$) at striatum level were probed with anti-pT89Prx2 antibody and rabbit Alexa Fluor 488 antibody (green) as secondary antibody. Relative amount of luminescence was assessed by normalizing the average intensity of signal emitted (20 counts/brain) in the endothelin-injected side to that of the noninjected side. Data are presented as mean $\pm$ SEM (*Student's *t*-test, $p < 0.05$).

emia. As shown in Figure 5d, the phosphorylation-resistant MEF2D significantly protected against damage and resulted in a smaller infarct (66% smaller) compared with GFP-injected control brains.

Overall, these results suggest that in an *in vivo* model of excitotoxic type of neuronal death cytoplasmic Prx2 and nuclear MEF2D are targeted and modulated by cdk5.

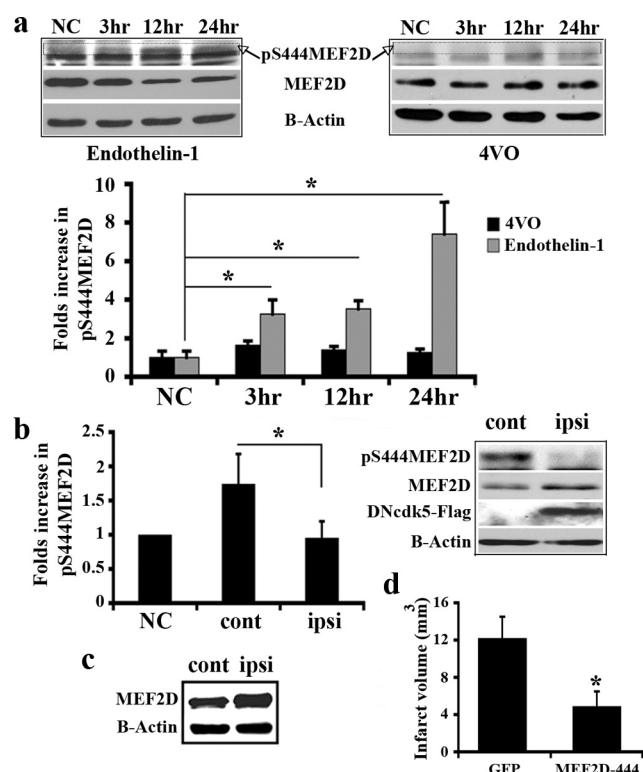


Figure 5. MEF2D is phosphorylated at Ser444 by cdk5 in focal ischemia. **a**, Analysis of pMEF2D level at the time points following endothelin-1 injection or 4VO. Total proteins extracted from striatum (focal ischemia) or hippocampus (4VO) were subjected to Western blot analysis using anti-pS444MEF2D and then MEF2D antibodies. The pMEF2D band has been marked in blots. The membrane is representative of $n = 3$ experiments and graph presents densitometry values of pMEF2D relative to MEF2D. **b**, Inhibition of cdk5 attenuates pMEF2D in focal ischemia. Rats were unilaterally injected with AAV expressing DNcdk5 into striatum and 14 d later endothelin-1 was injected into both sides. Proteins from both sides of the striatum were analyzed for pMEF2D by Western blot at 24 h after ischemia. Expression of DNcdk5 has been shown using anti-Flag antibody. The membrane is representative of $n = 3$ experiments and graph presents densitometry values of pMEF2D relative to MEF2D. **c**, Expression of AV-mediated MEF2D-444 in striatum was detected by Western blot analysis. **d**, Quantification of infarct volume in brains overexpressing GFP ($n = 4$) and MEF2D-444 ($n = 5$) following focal ischemia. Data are presented as mean $\pm$ SEM (*Student's t -test, $p < 0.05$).

Discussion

Although cdk5 has received considerable attention as a potential mediator of neuronal damage, evidence for its functional importance in adult models of injury, including stroke, has only recently begun to emerge (Smith et al., 2003; Wang et al., 2003; Rashidian et al., 2005). However, the essential role of cdk5, with respect to its localization and targets, in regulating ischemic damage is not completely clear. Our present work is significant because we have shown the following: (1) we have defined the cytoplasmic and nuclear compartments in which cdk5 acts to promote ischemic death; (2) we show that the involvement of these cellular compartments is different depending upon the particular model. This highlights the important notion that one mechanistic paradigm does not describe the mode of action of cdk5; (3) we define Prx2 and MEF2D as critical substrates for cdk5.

Differential roles of cdk5: importance of cytoplasmic cdk5 in ischemic damage

Cdk5 displays two opposing and seemingly contradictory functions. It is a key regulator of neuronal morphology and its activity

is essential during development (Dhavan and Tsai, 2001). In contrast, several lines of evidence have suggested that it could also be essential in neuronal death (Bu et al., 2002; Nguyen and Julien, 2003; Smith et al., 2003; Cruz and Tsai, 2004; Rashidian et al., 2005). The manner by which a key regulator of proper neuronal function can also possess a pathogenic role has been of considerable interest. While several possibilities might account for this contradiction, cell localization may be critical. In this regard, cdk5 activity was initially thought to be predominately localized membranous compartments. Presumably, these membrane-bound cdk5 complexes fulfilled the “normal” function of cdk5. In support of this, we have shown that inhibition of cytoplasmic cdk5 actually promotes death following DNA damage *in vitro* (O'Hare et al., 2005). In contrast, a pathogenic form of cdk5 was proposed to occur through calpain-mediated cleavage of the activator to a shorter more stable form. The hyperactivity of this complex was presumed to be deleterious. This cleavage also deletes the myristoylation sequence allowing cdk5 to traffic abnormally to the nucleus (Patrick et al., 1999; Kusakawa et al., 2000). In support of this, a recent report indicated that nuclear cdk5 activity increases in response to glutamate and that a nuclear target of cdk5, MEF2D, is functionally relevant for excitotoxic death, at least *in vitro* (Tang et al., 2005). These observations have led to a simplistic model by which normal cdk5 activity is membrane bound while pathogenic cdk5 is nuclear. However, the situation is likely much more complex. For example, recent reports have suggested that cdk5/p35 complex can normally be soluble and displays higher activity than membranous complex (Sato et al., 2007). Moreover, p35 can be specifically imported into nucleus (Fu et al., 2006). In addition, whether the model described above holds true in all cases, particularly following adult injury, is unclear. Indeed, one important possibility is that cdk5 complexes may also play a role in death/survival in the cytoplasm. Surprisingly, our results indicate that cytoplasmic cdk5 appears to play a crucial role in promoting death following ischemia. This contrasts with our previous findings that cytoplasmic cdk5 functions to promote survival following DNA damage (O'Hare et al., 2005) or that nuclear cdk5 activity is critical in promoting excitotoxic death (O'Hare et al., 2005; Tang et al., 2005). These latter studies were performed *in vitro* and suggest that the function of cdk5 is critically context-dependent and may differ in the adult. These data are also critical in that they show that cdk5 in the nucleus does not necessarily promote death. Indeed, under select conditions (i.e., global ischemia) we could not detect robust nuclear cdk5 activation. In models of focal ischemia, in contrast, activation and requirement of cdk5 activity is found in both nuclear and cytoplasmic fractions. The reasons for these differences are unclear, but likely are mediated through the specifics of the signaling environment for each ischemic model. It is clear that the activation of cdk5 is not solely mediated through formation of p25 in either global or focal model (data not shown). Indeed, we have noticed that p25 is formed in both fractions, but that there is no correlation between the timing of the formation of p25 and the induction of cdk5 activity (data not shown). This indicates that other signals (type of cells, other activators) are involved to determine the extent of cdk5 activation.

Cytoplasmic target for cdk5 in stroke

How does cdk5 induce its death signal within cytoplasm? Presently, we describe a new cytoplasmic target for cdk5 in stroke, the antioxidant enzyme Prx2. Oxidative stress is a critical mediator of death in stroke, and management of ROS has been shown to improve stroke in a number of stroke contexts (Margaill et al., 2004).

2005). Prx2, a cytoplasmic antioxidant enzyme with peroxidase activity, is abundantly expressed in neurons (Jin et al., 2005) and likely a major candidate in the defense of the neurons against oxidative stress. We have recently provided evidence that Prx2 is a substrate for cdk5 (Qu et al., 2007). We showed that Prx2 can be phosphorylated by cdk5 on Thr89 and that this modification leads to its inactivation (Qu et al., 2007). Presently, we demonstrate upregulation of Prx2 phosphorylation on Thr89 following focal and global ischemia in a manner dependent upon cdk5. We also show that modulation of Prx2 levels regulates death providing evidence for a functional importance of this activity. Interestingly, Prx2 has been implicated in other neurodegenerative situations. For example, Prx2 was found coaggregated with GPx1 and SOD-1 in neuronal Lewy body-like hyaline inclusions of spinal cord of familial ALS (Kato et al., 2004). We also demonstrated that Prx2 is phosphorylated in PD (Qu et al., 2007). Therefore, modulation of Prx2 levels may be a common and critical factor in the management of free radical stress that is critical for death regulation.

In addition to Prx2, there are likely other cytoplasmic targets of cdk5 in stroke. Potential targets include tau (Patrick et al., 1999; Cruz and Tsai, 2004). For example, tau has been proposed to be phosphorylated at Ser199/202 by cdk5 following transient forebrain ischemia (Morioka et al., 2006). However, this observation was supported by using a relatively nonspecific CDK inhibitor, olomoucine. Therefore, it is unclear whether other CDKs may account for this phosphorylation activity. In addition, the actual role of tau phosphorylation in this case, is unclear. A second target also includes the NMDA receptor NR2A subunit. Cdk5 is reported to phosphorylate this subunit leading to increased calcium influx and neuronal death molecular cascades (Li et al., 2001). Understanding of whether/how these multiple targets interact will be of interest in future studies.

Nuclear target for cdk5 in stroke

In addition to its role in the cytoplasm, cdk5 may also have additional roles in the nucleus. In fact, several targets, which are predominately nuclear, have been described. These include the tumor suppressor p53 and the transcription factor MEF2D. For example, under select apoptotic conditions, cdk5 has been shown to increase p53 levels (Lee et al., 2007). Cdk5 has also been shown to inactivate survival factors such as MEF2D. In this case, cdk5 phosphorylates MEF2D on Ser444 suppressing its transcriptional activity. This Ser444 phosphorylation has been shown to occur in *in vitro* models of death following peroxide and excitotoxicity exposure in a manner dependent on cdk5 (Gong et al., 2003; Tang et al., 2005). However, its role in adult models of stroke *in vivo* was unknown. Presently, we show that MEF2D is also phosphorylated on Ser444 in a focal ischemic model where nuclear cdk5 activity is observed, but not following global insult where nuclear cdk5 activity is not detected. Consistent with this observation, we also show that restoration of active form of MEF2D is protective following focal ischemia. While our results suggest the possibility that modulation of MEF2D by itself is sufficient to account for the death-promoting effects of MEF2D in the nucleus, there may be additional nuclear targets of cdk5 in stroke. In this regard, it will be interesting to examine other potential targets of cdk5, such as p53, which has also been implicated in stroke (Crumrine et al., 1994).

In summary, we have described the critical nature of cytoplasmic cdk5-Prx2 activity in modulating ischemic neuronal death and context-dependent involvement of a nuclear cdk5-MEF2D pathway. These results also indicate the complex nature of cdk5

signaling in neuronal injury, suggesting that the nuclear cdk5 activity is not necessarily required for death.

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

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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- κ B) pathway as Pim-1 expression and activity are significantly blocked by siRNA-mediated knockdown of NF- κ B or NF- κ B 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- κ B, 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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Abbreviations used: ATM, ataxia telangiectasia mutated; CAPE, caffeic acid phenethyl ester; Cdc, cell division cycle; Cdk, cyclin-dependent kinase; Chk1, checkpoint kinase 1; IP, immunoprecipitation; JNK, c-Jun N-terminal kinase; NF- κ B, 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- κ B) 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 phenethyl ester) 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 μ M) or NF- κ B inhibitors as indicated in the text and figures. Lipofectamine mediated transfection of siRNA for NF- κ B have been described previously (Aleyasin *et al.* 2004). siRNAs used in Fig. 7 to (siRNAp65, AAGAAGCACAGAUACCACCAA) and (control siRNA, GCGCGCUUUGUAGGAUUCG) 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'-GGCGCCGGGCAAAGAGAAGGAG-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 $\times$ 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 μ L 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 μ L of the sample were used as the input chromatin. Immunoprecipitation was performed overnight with p65 antibody and rabbit IgG mentioned above, then 50 μ L 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'-AACCTAGGATTTCTTCTGT-3' and 5'-CATGCACCTGTGTCACCA-3'. Human promoter sequences are also as follows: 5'-AGCCGACTTGGAGGTCTCGGGT-3' and 5'-GCTGTAGGCTGGGGCGGATTCTG-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 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 μ M) 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

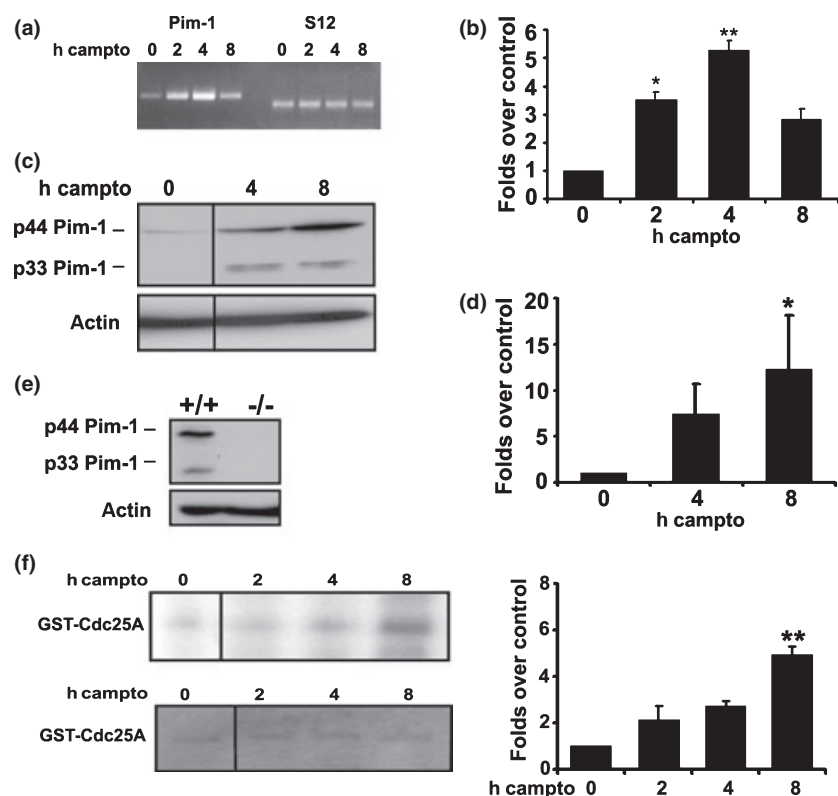


Fig. 1 Pim-1 activation in response to DNA damage. (a) Pim-1 mRNA increases early in response to camptothecin treatment. Total RNA was extracted from cortical neurons at the indicated times after camptothecin treatment (10 μ M) and analyzed for Pim-1 mRNA expression using RT-PCR. S12 levels are shown 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 $\pm$ 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 μ M) 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 $\pm$ 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 -³²P]ATP onto GST-Cdc25A substrate and subtracting the control background value. Left panel, an example of autoradiogram showing the incorporation of [γ -³²P]ATP onto Cdc25A. Right panel, the kinase activity was quantified by densitometry using NIH ImageJ. Data represent the mean $\pm$ 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 μ M) (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

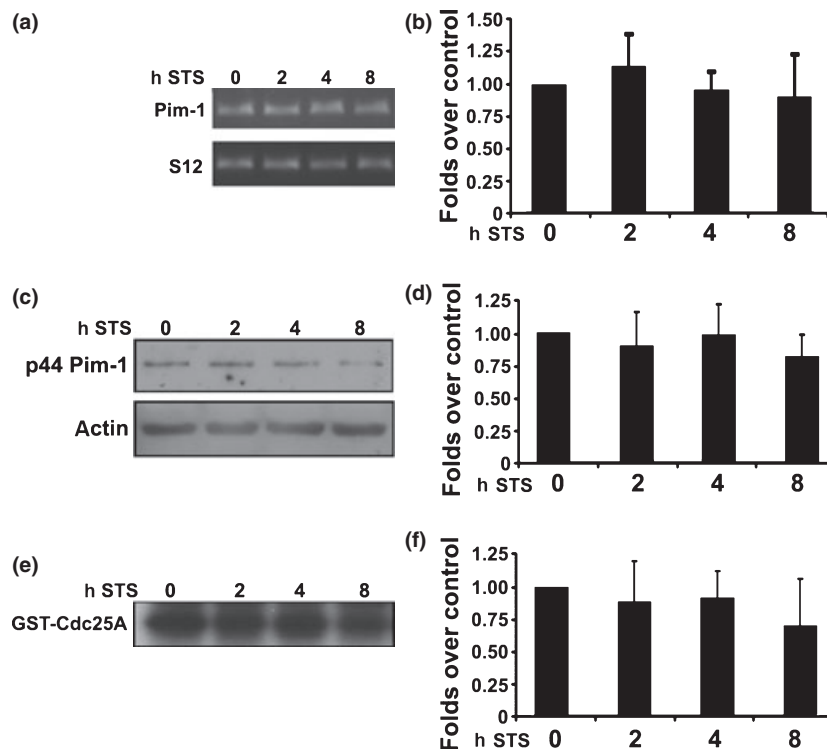


Fig. 2 Pim-1 is not activated in response to staurosporine. (a) Pim-1 mRNA does not increase in response to staurosporine (STS) treatment. Total RNA was extracted from cortical neurons at the indicated times after staurosporine treatment (1 μ M) and analyzed for Pim-1 mRNA expression using RT-PCR. S12 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 $\pm$ 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 μ M) 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 $\pm$ 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 $\pm$ 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 extracts. Cdc25A was immunoprecipitated using an anti-Cdc25A antibody and co-immunoprecipitated proteins were examined for the

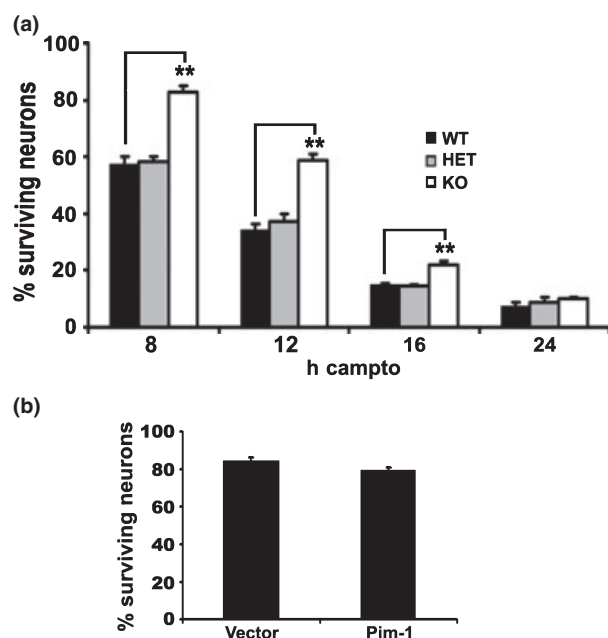


Fig. 3 (a) Pim-1 deficiency is protective against camptothecin-induced neuronal death. Cortical neurons were obtained from E15 embryos derived from a double heterozygote breeding of Pim-1 mice. Cultures were exposed to camptothecin (10 μM) for 8, 12, 16 and 24 h and cell viability was analyzed by nuclear counts. ** $p < 0.01$ compared with control. Each data point is the mean $\pm$ 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- κ B 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- κ B in CD40 signaling in B lymphocytes (Zhu *et al.* 2002). This is consistent with the observation that the Pim-1 promoter (both human and mouse) contains several putative NF- κ B binding sites situated closely together (Fig. 5a). Accordingly, we first examined whether NF- κ B members can potentially bind these sites. We designed primer sequences (using DNASTAR software) close to the NF- κ B 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- κ B 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- κ B 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- κ B 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- κ B. To this end, we employed two different

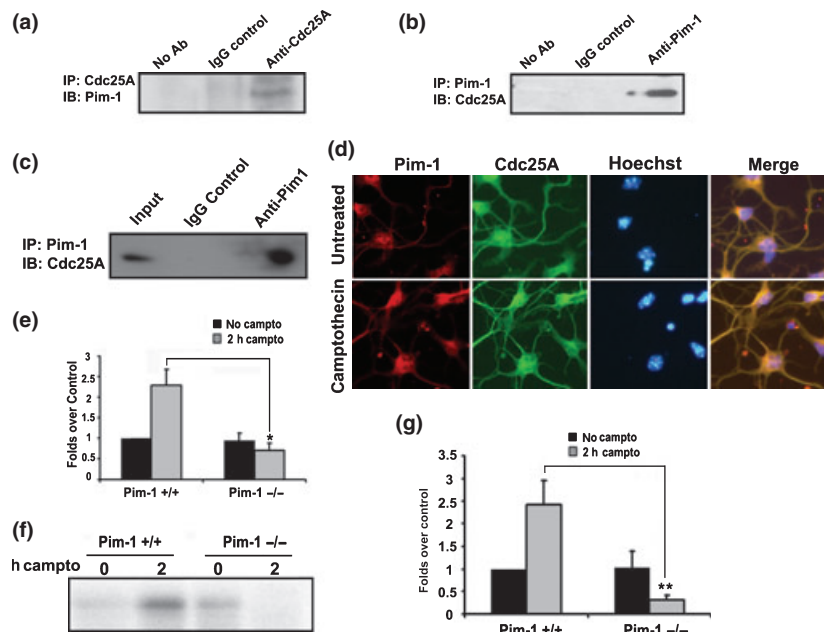


Fig. 4 Pim-1 regulates Cdc25A in response to DNA damage. (a and b) *In vivo* association of Pim-1 and Cdc25A. IP-western blot analysis of mouse brain extracts immunoprecipitated with anti-Cdc25A (or anti-Pim-1) antibody. Immunoprecipitates were run on SDS-polyacrylamide gel electrophoresis followed by 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 phos-

phatase 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 $\pm$ 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-associated kinase activity. The kinase activity was quantified by measuring the incorporation of [γ -³²P]ATP onto pRb substrate and subtracting the wild-type control background value. Data represent the mean $\pm$ SEM from five independent experiments. ** p < 0.01.

approaches to inhibit NF- κ B activation and observe whether camptothecin-evoked up-regulation of Pim-1 is affected. First, we used several different pharmacological inhibitors to block NF- κ B activity via different mechanisms: (i) CAPE inhibits nuclear translocation of NF- κ B possibly via antioxidant effects (Natarajan *et al.* 1996); (ii) Helenalin blocks DNA binding of NF- κ B by specific and irreversible alkylation of the p65 subunit (Lyss *et al.* 1997, 1998); (iii) BAY 11-7082 inhibits I κ B α phosphorylation and degradation thereby results in the inactivation of NF- κ B (Pierce *et al.* 1997). We treated cortical neurons with camptothecin (10 μ M) 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- κ B inhibitors significantly decreases Pim-1 signals at 4 h after camptothecin treatment. Similarly, co-treatment of NF- κ B 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- κ B and Pim-1 using siRNA-mediated knockdown approach. In this regard, we transfected neurons with two independent NF- κ B-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- κ B 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- κ B, as a pro-apoptotic factor, is

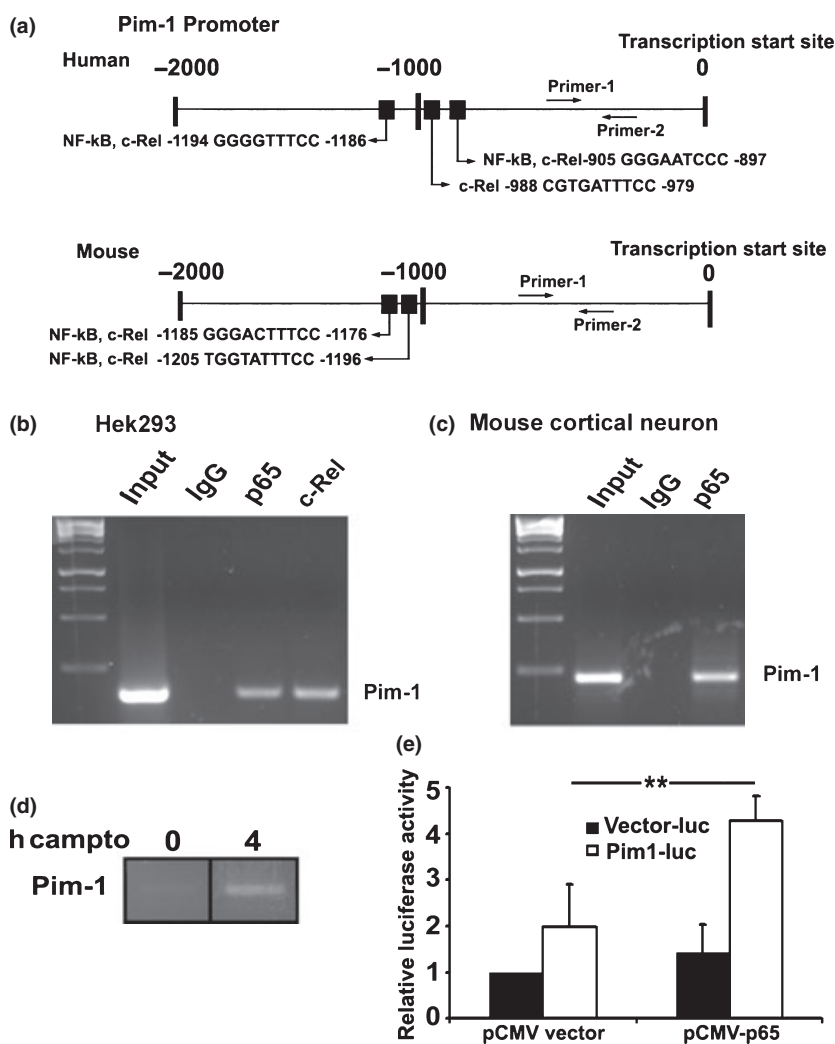


Fig. 5 NF-κB interacts with Pim-1 promoter in both mouse and human cells. (a) NF-κB binding sites in Pim-1 promoter region as identified through the TFSEARCH server (<http://www.cbrc.jp/research/db/TFSEARCH.html>). (b) ChIP assay to show p65 and c-Rel subunits bind to Pim-1 promoter in HEK293 cells. (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 Lipofectamine 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-deficient 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

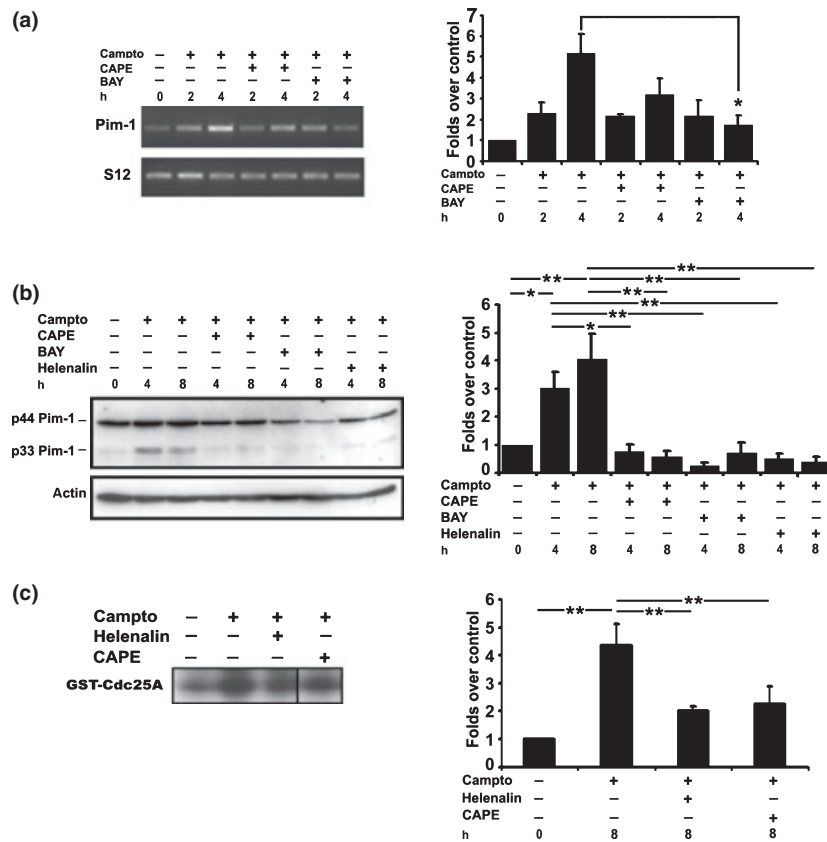


Fig. 6 NF- κ B inhibition blocks DNA damage-induced up-regulation of Pim-1. (a) Cortical neurons were treated with camptothecin (10 μ M) alone or camptothecin plus CAPE (5 μ M) and BAY 11-7082 (5 μ M) for 2 and 4 h. Total RNA was extracted and Pim-1 transcripts were analyzed by RT-PCR. S12 was analyzed as a control (left panel). Densitometric analysis of RT-PCR was performed from three independent experiments (right panel). Data represent the mean $\pm$ SEM from three independent experiments. * p < 0.05. (b) Cortical neurons were treated with camptothecin (10 μ M) alone or camptothecin plus CAPE (5 μ M), BAY 11-7082 (5 μ M) and Helenalin (5 μ M) 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 $\pm$ SEM from three independent experiments. * p < 0.05, ** p < 0.01. (c) NF- κ B inhibition blocks Pim-1 kinase activity induced by DNA damage. Cortical neurons were treated with camptothecin (10 μ M) alone or camptothecin plus Helenalin (5 μ M) or CAPE (5 μ M) 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 $\pm$ SEM from three independent experiments. * p < 0.05.

which is regulated by NF- κ B 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- κ B) 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

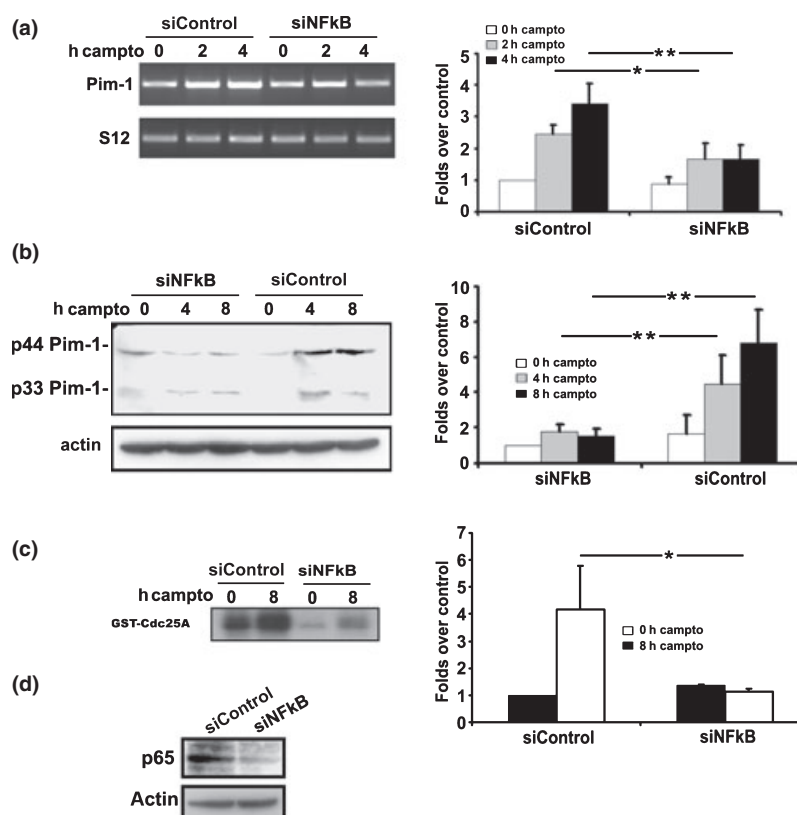


Fig. 7 NF- κ B knockdown blocks DNA damage-induced up-regulation of Pim-1. (a) Cortical neurons were transfected with a control siRNA (siControl) or NF- κ B siRNA oligonucleotides (siNF- κ B) for 24 h and then treated with camptothecin for 2 and 4 h as indicated. Pim-1 transcripts were analyzed by RT-PCR (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 $\pm$ 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- κ B p65 subunit in cells treated with NF- κ B 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

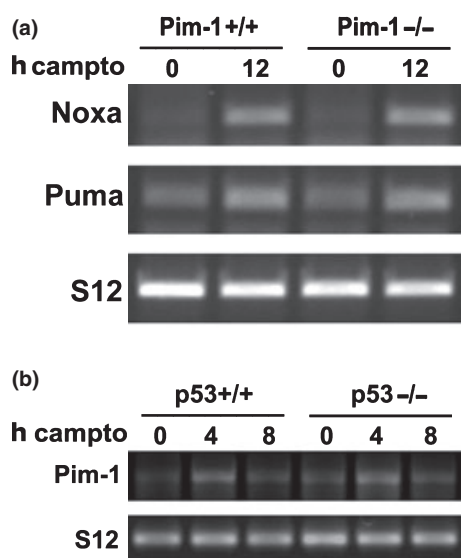


Fig. 8 (a) Pim-1 deficiency does not inhibit the p53-inducible genes Noxa and Puma. Cortical neurons from Pim-1^{+/+} and Pim-1^{-/-} mice were treated with and without camptothecin for 12 h. Noxa and Puma levels were then analyzed by RT-PCR as described in Material and Methods. S12 was also analyzed 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- κ B plays a dual role in regulating neuronal loss where basal NF- κ B activity is critical for survival while acute NF- κ B activation signals death through induction of p53 message (Aleyasin *et al.* 2004). It is important to point out that NF- κ B, 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- κ B is also critical for induction of Pim-1 message/protein which in turn is critical for Cdk activation. This evidence suggests that NF- κ B 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- κ B/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 Cdk4s are activated, particularly in an *in vivo* context. Interestingly, NF- κ B 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 Cdk4s 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 Cdk4s, 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- κ B. NF- κ B also works in conjunction with other regulators such as ATM and calpains to regulate p53 activation. Cdk4s 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). Cdk4s 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 Cdk4s (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.

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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- κ B down-regulation blocks DNA damage-induced up-regulation of Pim-1.

Appendix S1. Supplementary Materials and Methods.

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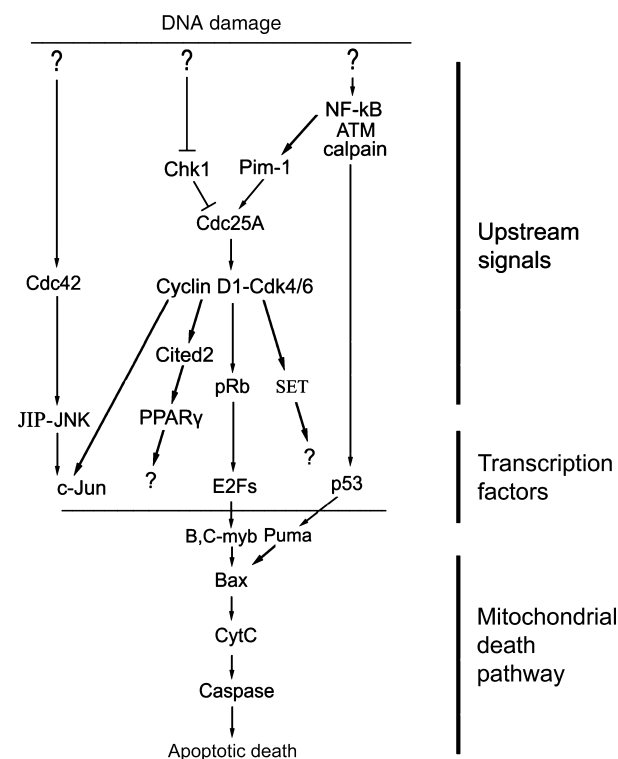


Fig. 9 Schematic model of the signaling pathways involved in DNA damage induced death.

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Huang E, Qu D, Venderova K, Zhang Y, Haque ME, **Rousseaux M**, Tandon A, Slack RS, Kim RH, Mak TW, Woulfe JM, Park DS. Apurinic/apyrimidinic Endonuclease 1 (APE1): a critical target of Cdk5 in neuronal death, *Nat. Cell. Biol.* 2010 Jun; 12(6):563-71

Contribution: My contribution to the research consisted of performing some *in vivo* experiments with animals of the DJ-1 colony, analyzing data and editing the manuscript/figures for publication. My overall contribution to this paper was 5-10%.

The role of Cdk5-mediated apurinic/aprimidinic endonuclease 1 phosphorylation in neuronal death

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Accumulating evidence suggests that deregulated cyclin-dependent kinase 5 (Cdk5) plays a critical part in neuronal death. However, the pathogenic targets of Cdk5 are not fully defined. Here we demonstrate that the Cdk5 activator p35 interacts directly with apurinic/aprimidinic endonuclease 1 (Ape1), a protein crucial for base excision repair (BER) following DNA damage. Cdk5 complexes phosphorylate Ape1 at Thr 232 and thereby reduces its apurinic/aprimidinic (AP) endonuclease activity. Ape1 phosphorylation is dependent on Cdk5 in *in vitro* and *in vivo*. The reduced endonuclease activity of phosphorylated Ape1 results in accumulation of DNA damage and contributes to neuronal death. Overexpression of Ape1^{WT} and Ape1^{T232A}, but not the phosphorylation mimic Ape1^{T232E}, protects neurons against MPP⁺/MPTP. Loss of Ape1 sensitizes neurons to death. Importantly, increased phosphorylated Ape1 was also observed in post-mortem brain tissue from patients with Parkinson's and Alzheimer's diseases, suggesting a potential link between Ape1 phosphorylation and the pathogenesis of neurodegenerative diseases.

Cdk5 is a Pro-directed Ser/Thr kinase that belongs to the cyclin-dependent kinase (Cdk) family^{1,2}. Growing evidence indicates that deregulated Cdk5 promotes neuronal death under conditions of stress^{3–7}. Deregulated Cdk5 is also implicated in the pathogenesis of neurodegenerative disorders such as Alzheimer's disease^{8–10} and Parkinson's disease¹¹. For example, we showed previously that loss of dopaminergic neurons induced by the neurotoxin MPTP is mediated by calpain-mediated cleavage of p35 to p25, and subsequent deregulation of Cdk5 activity^{3,11,12}. However, the identity of Cdk5 substrates affected under these pathogenic conditions remains unclear. We therefore performed a mass spectrometry-based interactomics screen and identified several potential p35-interacting proteins¹¹. Recently, we reported on one such target, peroxiredoxin-2 (Prx2), a crucial cytoplasmic target of the p35/p25/Cdk5 complexes¹¹. We showed that its peroxidase activity is reduced by Cdk5-mediated phosphorylation and that this reduction participates in loss of dopaminergic neurons. We now report identification of Ape1, a nuclear substrate of p35/p25/Cdk5 complexes.

Ape1 is a central enzyme in the BER pathway, which is crucial for the repair of DNA damaged by oxidative stress and other genotoxins^{13–15}. In the BER pathway, the AP endonuclease activity of Ape1 cleaves AP sites (a major type of oxidative DNA damage), which are then processed by other enzymes to repair the damaged DNA^{13–15}. Ape1 also functions as a redox factor, maintaining transcription factors in their reduced state, a

prerequisite for transcription^{13–15}. There is evidence that Ape1 may have a role in cell function and survival through its DNA-BER activity^{16–19}. The functional importance of this protein is further underscored by findings that mice nullizygous for *Ape1* gene are embryonic lethal at embryonic day 5.5 (ref. 20).

Here we define a critical pathway by which Cdk5 mediates neuronal loss. We demonstrate that Cdk5/p35 complexes interact directly with Ape1 and phosphorylate Ape1 at Thr 232, which reduces its AP endonuclease activity and consequently contributes to MPP⁺/MPTP-induced neuronal death and accumulating DNA damage.

RESULTS

Cdk5/p35 complexes interact with Ape1 and phosphorylate Ape1

Data from a previous mass spectrometry-based interactomics screen¹¹ indicated that Ape1 may interact with p35. To determine whether p35 interacts directly with Ape1 *in vitro*, we demonstrated, with an *in vitro* binding assay, that GST–Ape1, but not GST control, precipitated His-tagged p35 (Fig. 1a), and that GST–p10 and p35, but not GST control, GST–p25 or GST–Cdk5, precipitated His–Ape1 (Fig. 1b). This indicates that the p10 fragment of p35 binds to Ape1.

We next examined whether endogenous Cdk5/p35 complexes interact with Ape1 *in vivo*. Ape1 in mouse brain extracts was immunoprecipitated with antibodies to p35 or Cdk5, but not with an IgG control (Fig. 1c, left).

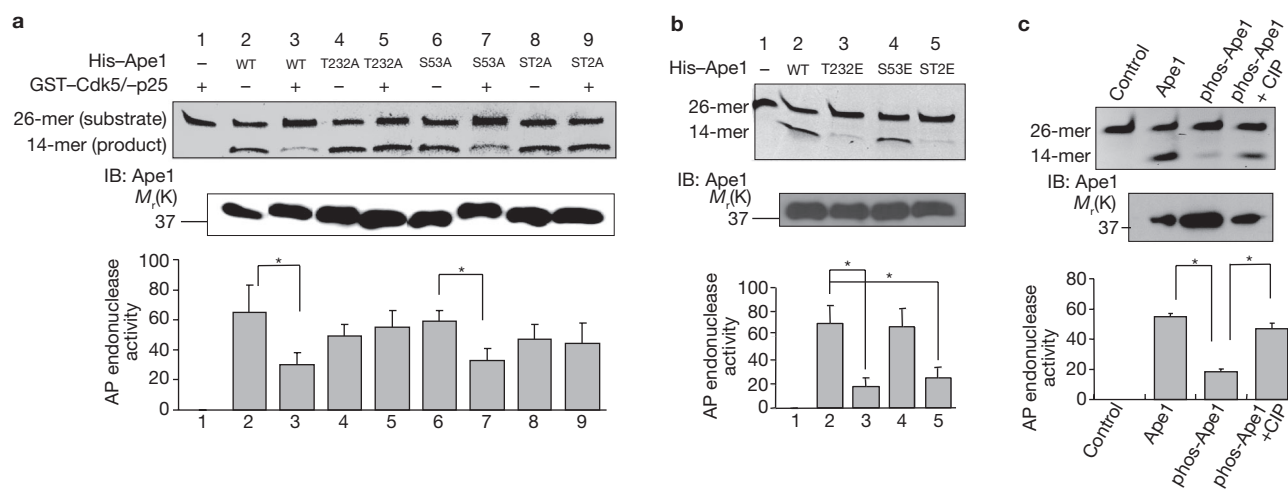


Figure 2 The effect of Cdk5-mediated phosphorylation of Ape1 and its mutants on AP endonuclease activity *in vitro*. (a) His-Ape1^{WT} (5 μ g) and non-phosphorylatable mutants S53A, T232A or S53A/T232A (ST2A) were incubated with or without GST-Cdk5 (0.2 μ g) and GST-p25 (0.2 μ g) in 500 μ l kinase assay buffer at 30 °C overnight. A sample of reaction mixture (1 μ l) was processed for the AP-endonuclease activity assay (top panel). (b) His-Ape1^{WT} (10 ng) or phosphorylation mimic mutants S53E, T232E or S53E/T232E (ST2E) were assayed by the AP endonuclease activity assay (top panel). (c) The effect of phosphatase treatment on AP endonuclease

activity of Ape1. His-Ape1 (5 μ g) was incubated with GST-Cdk5/p25 (0.2 μ g) at 30 °C overnight and then treated with or without calf intestinal phosphatase (CIP, 10 unit ml⁻¹) and roscovitine (10 μ M) for 3 h and processed to AP-endonuclease activity assay (top panel). Control without any proteins to show uncleaved substrate (26-mer). The middle panels in a–c shows the western blots for Ape1 of the reaction mixture (20 μ l). The bottom panels in a–c shows quantification of AP endonuclease activity of proteins. The AP endonuclease activity was calculated as product/(product + substrate) $\times$ 100 (n = 3). Error bars represent s.e.m.

Ape1^{T232A} or Ape1^{ST2A} with GST-Cdk5/p25 (Fig. 2a) or GST-Cdk5/p35 (Supplementary Information, Fig. S2a) did not significantly affect the AP endonuclease activity. Consistently, the AP endonuclease activity of the phosphorylation mimic mutants His-Ape1^{T232E} or His-Ape1^{ST2E}, but not His-Ape1^{S53E}, was significantly lower (~75% or ~63%) than that of His-Ape1^{WT} (Fig. 2b). Dephosphorylation of Ape1 by calf intestinal phosphatase (CIP, Supplementary Information, Fig. S2b) rescued its AP endonuclease activity by 86% (Fig. 2c). These results indicate that Cdk5-mediated Ape1 phosphorylation at Thr 232, but not Ser 53, reduces its AP endonuclease activity *in vitro*.

Ape1 phosphorylation at Thr 232 is induced in neurons after MPP⁺ treatment

We next examined whether the Thr 232 of Ape1 was endogenously phosphorylated by activated Cdk5 in cortical neurons treated with MPP⁺ (1-methyl-4-phenylpyridinium), which results in apoptotic-type death (Supplementary Information, Fig. S3a, b). First, we generated an antibody, pT232, specific for Ape1 phosphorylated at Thr 232 (phos-Ape1). The pT232 antibody specifically recognized the phos-Ape1 but not native Ape1 (Fig. 3a). The phosphorylation signal was blocked by pre-incubation with phosphopeptide (p-peptide) antigen (CNAGFpT²³²PQER; Fig. 3a, right) but not with the homologous non-p-peptide (CNAGFT²³²PQER; Fig. 3a, left). In addition, CIP treatment reversed phosphorylation by Cdk5/p25, and His-Ape1^{T232A} was resistant to phosphorylation (Supplementary Information, Fig. S2b). These data confirm the specificity of the pT232 antibody.

The level of phos-Ape1 and the ratio of phos-Ape1 to total Ape1 in cultured cortical neurons were increased and peaked 12 h after MPP⁺ treatment (20 μ M; Fig. 3b). Similar results were obtained in neurons treated with methanesulphonate (MMS), a DNA damaging agent (Supplementary Information, Fig. S3c, d), indicating that Ape1 is responsive to oxidative stress and DNA damage. We also showed

that DNA polymerase β (DPOLB), but not 8-oxoguanine DNA glycosylase (Ogg1), was induced in MPP⁺-treated neurons (Supplementary Information, Fig. S3e, f).

Immunofluorescence analysis also showed that 12 h after MPP⁺ treatment, both phos-Ape1 and total Ape1 were induced in cortical neurons, and that the increased signal density of phos-Ape1 (85% increase) was more than that of total Ape1 (38% increase) after MPP⁺ exposure (Fig. 3c, d; Supplementary Information, Fig. S3g). The fluorescence signal of phos-Ape1 was blocked by pre-incubation with the p-peptide (Fig. 3cC, cD). Importantly, western blot analyses of total and/or nuclear extracts show that the major band that seems to increase after MPP⁺ treatment is the one associated with the molecular weight of Ape1 (~35K; Supplementary Information, Fig. S3h). These data again support the specificity of the observed phospho-signal. Interestingly, consistent with the effects of p10 on Cdk5-mediated phosphorylation *in vitro* (Supplementary Information, Fig. S1c, d), overexpression of p10 also reduced the phos-Ape1 signal in MPP⁺-treated neurons (Supplementary Information, Fig. S4a, b).

Ape1 phosphorylation at Thr 232 after MPP⁺ treatment is mediated specifically by Cdk5

We further determined, by western blotting, whether the phosphorylation of Ape1 is mediated specifically by Cdk5 in MPP⁺-treated neurons. Induction of phos-Ape1, but not total Ape1, was blocked by roscovitine (10 μ M) 12 and 24 h after MPP⁺ treatment (Fig. 3e). Because roscovitine inhibits not only Cdk5 but also other Cdks, we also examined the phosphorylation in *p35*^{-/-} neurons, which are deficient in Cdk5 activity²¹. The induction of phos-Ape1 was observed in cortical neurons of *p35*^{+/+}, but not *p35*^{-/-}, littermates 12 h following MPP⁺ exposure (Fig. 3f). Similar findings were observed by immunofluorescence analyses (Supplementary Information, Fig. S4c–e). Together, these data indicate that Ape1 phosphorylation at Thr 232 is mediated specifically by Cdk5.

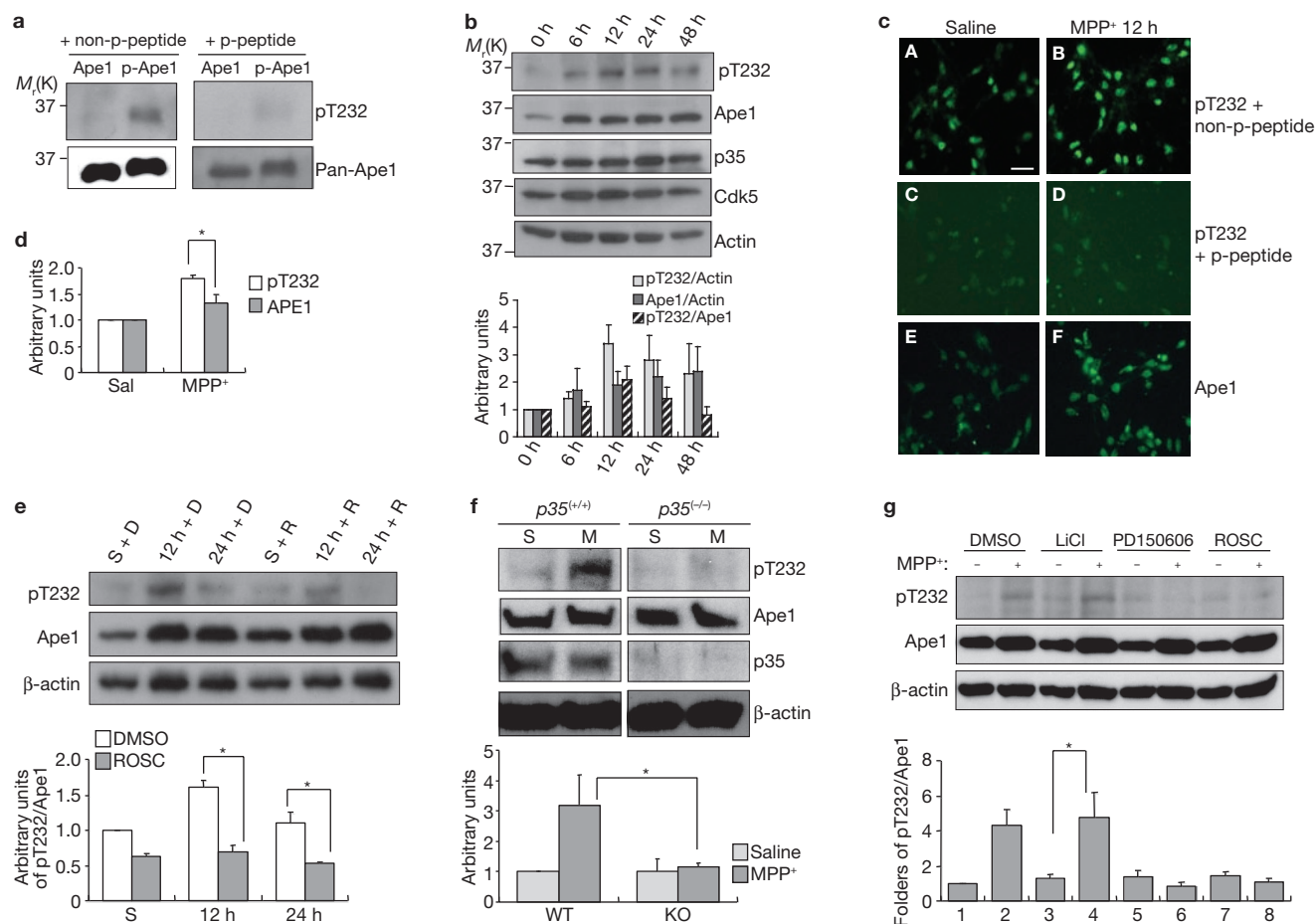


Figure 3 Induction of Ape1 phosphorylation at Thr 232 in MPP⁺-treated neurons. **(a)** Determination of specificity of the anti-phos-Ape1 at Thr 232 (pT232) antibody. Ape1 (1 μg) or phos-Ape1 (1 μg) produced by incubating His-Ape1 with Cdk5/p25, were probed by western blot analyses using the pT232 antibody pre-incubated with non-p-peptide (CNAGFT²³²PQER) or p-peptide antigen (CNAGFP²³²PQER) used to produce the antibody. A pan-Ape1 antibody was used as a loading control. **(b)** Increase of phos-Ape1 in MPP⁺-treated neurons. Cultured cortical neurons were treated with MPP⁺ (20 μM) for 0, 6, 12, 24 or 48 h and were analysed by western blot analyses using pT232, then reprobed with indicated antibodies (top panel). The bottom panel in **b** shows quantification of the ratio of densitometric values between pT232, total Ape1 and β-actin from western blot (n = 3). Error bars represent s.e.m. **(c)** Increase of phos-Ape1 in cortical neurons 12 h after MPP⁺ treatment (MPP⁺ 12 h) analysed by immunofluorescence staining. Neurons were incubated with: pT232, pre-incubated with the non-p-peptide (CNAGFT²³²PQER) (A, B); pT232, pre-incubated with the

p-peptide (p-peptide, CNAGFP²³²PQER) (C, D); anti-Ape1 antibody for immunofluorescent staining (E, F). Scale bar, 20 μm. **(d)** Quantification of fluorescence by image analysis signal density from neurons in each group (n = 3). Error bars represent s.e.m. **(e)** Cortical neurons were pre-treated with DMSO (D) or roscovitine (R, 10 μM) for 3 h and then treated with saline (S) for 12 h as a control or MPP⁺ (20 μM) for 12 or 24 h. **(f)** Neurons from *p35*^{+/+} or *p35*^{-/-} embryos were treated with saline or MPP⁺ for 12 h. **(g)** Cortical neurons were pre-treated with DMSO, LiCl (10 mM), PD150606 (100 μM) or roscovitine (10 μM) and then treated with saline or MPP⁺ for 12 h. The top panels in **e–g** show analysis of cell lysates by western blot using indicated antibodies. The separated splices came from the same film of western blotting and derived from the same set of measurements **(g)**. The bottom panels in **e–g** show quantification of the densitometric values of pT232 relative to Ape1 from western blots (n = 3). Error bars represent s.e.m. Uncropped images of the western blots are shown in Supplementary Information, Fig. S8 (b, e–g).

Finally, we also showed that a GSK3 inhibitor, lithium chloride (LiCl), did not affect the induction of phos-Ape1 in MPP⁺-treated neurons, whereas a calpain inhibitor PD150606 or roscovitine blocked the induction (Fig. 3g). This indicates that calpains, but not GSK3, are involved in the signalling pathway that leads to phosphorylation of Ape1.

Inhibition of Cdk5 rescues AP endonuclease activity and reduces DNA damage

We next examined whether the endogenous Ape1 phosphorylation by Cdk5 can reduce its AP endonuclease activity in MPP⁺-treated neurons. We observed an increase in AP endonuclease activity in cortical neurons 12 and 24 h after MPP⁺ treatment (Fig. 4a). This

may be attributable to induction of total Ape1 after MPP⁺ exposure. Importantly, however, roscovitine treatment resulted in a further substantial increase in AP endonuclease activity (Fig. 4a). We also observed that the AP endonuclease activity was elevated in *p35*^{-/-} cortical neurons, compared with *p35*^{+/+}, after MPP⁺ treatment (Fig. 4b). These data indicate that inhibition of Cdk5 activity leads to a significant increase in AP endonuclease activity in MPP⁺-treated neurons.

We also determined whether augmentation of AP endonuclease activity mediated by Cdk5 inhibition contributes to DNA repair. The number of AP sites was significantly elevated in neurons 12 and 24 h after exposure to MPP⁺, and this increase was reduced after roscovitine

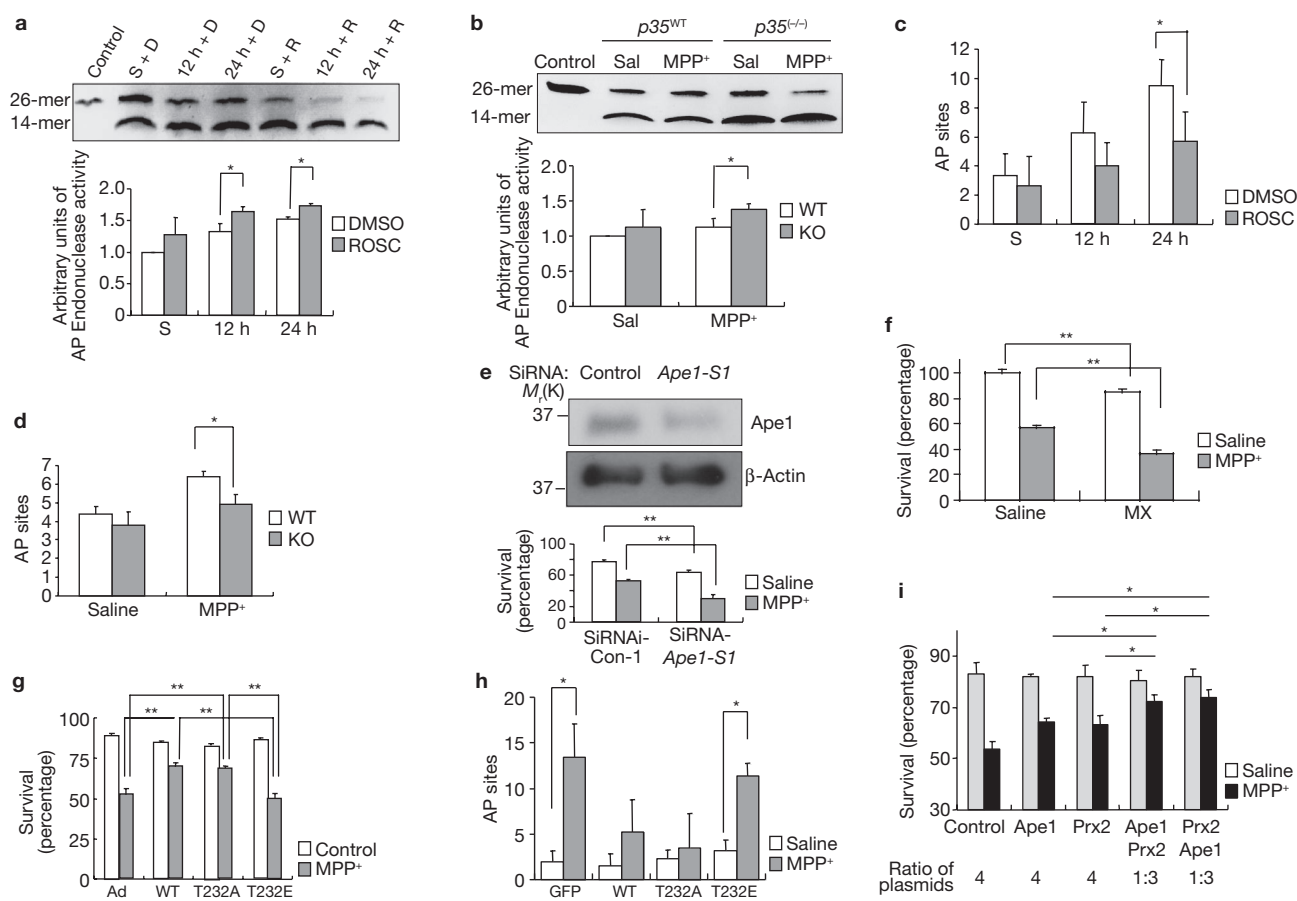


Figure 4 The role of Ape1 phosphorylation at Thr 232 in MPP⁺-treated neurons. (a) Cortical neurons were treated with saline or MPP⁺ (20 μM) for 12 or 24 h with DMSO (D) or 10 μM roscovetine (R). Samples were processed by the AP endonuclease activity assay. Control: without any cell extract (top panel). Quantification of AP endonuclease activity ($n = 3$; bottom panel). (b) Neurons from *p35*^{WT} (WT) or *p35*^{-/-} (KO) embryos were treated with saline or MPP⁺ for 12 h. Samples were subjected to AP endonuclease assay (top panel). Quantification of AP endonuclease activity ($n = 3$; bottom panel). (c) Cortical neurons were treated with saline or MPP⁺ (20 μM) for 12 or 24 h with DMSO or roscovetine (ROSC, 10 μM). Cellular genomic DNA (1 μg) was subjected to AP sites assay ($n = 3$). (d) Cortical neurons from *p35*^{WT} or *p35*^{-/-} embryos were treated with saline or MPP⁺ for 12 h. Cellular genomic DNA (1 μg) was subjected to AP sites assay ($n = 3$). (e) Cortical neurons were transfected with *Ape1-S1* siRNA for 48 h, and then analysed by western blot

using anti-Ape1 antibody (top panel), and evaluated by neuronal survival assay ($n = 3$; bottom panel). (f) Cortical neurons were pre-incubated with methoxamine (MX, 25 mM; pH adjusted to 7.4). Neuronal survival after MPP⁺ or saline treatment was then quantified ($n = 3$). (g) Cortical neurons were transfected with pAdtrack-CMV plasmids expressing GFP, Ape1^{WT}, Ape1^{T232A} or Ape1^{T232E}. Neuronal survival after MPP⁺ or saline treatment was evaluated ($n = 3$). (h) Cortical neurons were infected with adenoviral vector expressing GFP, Ape1^{WT}, Ape1^{T232A} or Ape1^{T232E} and then exposed to MPP⁺ (20 μM) or saline for 48 h. Cellular genomic DNA (1 μg) was subjected to AP sites assay ($n = 3$). (i) Cortical neurons were transfected with plasmids expressing GFP alone (4 μg/well), Ad-Ape1 (pAdtrack-CMV, 4 μg/well), Ad-Prx2 (pAdtrack-CMV, 4 μg/well), and co-transfected Ad-Ape1 with pCMV-Prx2 (1:3 μg/well ratio) or Ad-Prx2 with pCI-Ape1 (1:3 μg/well ratio). Neuronal survival was evaluated after MPP⁺ or saline treatment ($n = 3$). Error bars represent s.e.m.

treatment (Fig. 4c). A reduction in the number of AP sites was also detected in *p35*^{-/-} cortical neurons 12 h after MPP⁺ treatment when compared with *p35*^{WT} neurons (Fig. 4d). Interestingly, another report suggests that the level of AP sites observed here does not kill dividing HE21 cells²². However, neurons are more sensitive to DNA damage. Indeed, we observe that MMS (200–500 μM) treatment efficiently kills neurons (Supplementary Information, Fig. S4f, g), whereas the same concentrations did not induce an appreciable level of cell death in HE21 cells²².

The Ape1 phosphorylation is crucial for neuronal death induced by MPP⁺

To examine the role of Ape1 and Ape1 phosphorylation in MPP⁺-treated neurons, we used two separate Ape1-specific siRNAs (*Ape1-S1* and *S2* siRNA). First, we confirmed that these two siRNAs both reduce the level of Ape1 in mouse cortical neurons (Fig. 4e;

Supplementary Information, Fig. S5a), but not in human HEK293 cells (Supplementary Information, Fig. S5b). Next, we observed that both siRNAs resulted in neuronal death with or without MPP⁺ treatment, as analysed by survival assay (Fig. 4e) and TUNEL staining (Supplementary Information, Fig. S5c). Finally, to further support the specific nature of siRNA downregulation, we observed that human Ape1 rescued the neuronal death induced by the *Ape1* siRNAs (Supplementary Information, Fig. S5c). Taken together, these data indicate that Ape1 has a protective role in this death paradigm. Moreover, siRNA-mediated downregulation of Ape1 also diminished the phos-Ape1 signal (Supplementary Information, Fig. S5d), confirming the specificity of the pT232 antibody.

To more specifically examine the endonuclease activity of Ape1, we used the Ape1 inhibitor methoxyamine¹⁵. This treatment also sensitized neurons to death with or without MPP⁺ treatment (Fig. 4f). Methoxyamine,

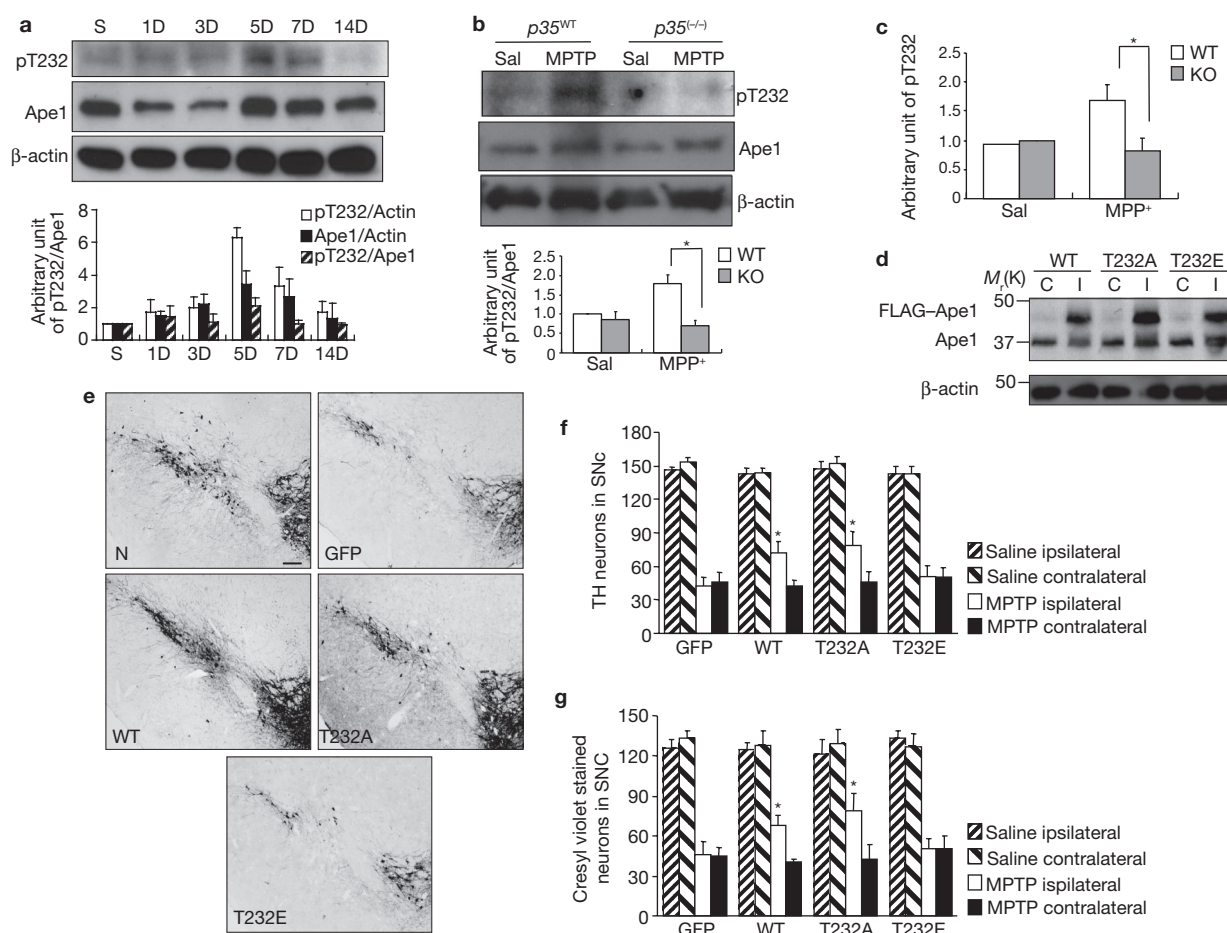


Figure 5 The role of Ape1 phosphorylation at Thr 232 in MPTP-injected mice and mediated by Cdk5. (a) The SNc extracts from mice after MPTP administration for the indicated time course were subjected to western blot using indicated antibodies (top panel). Quantification of the ratio of densitometric values between pT232, total Ape1 or actin from western blots ($n = 3$; bottom panel). (b) The SNc extracts from $p35^{+/+}$ (WT) or $p35^{-/-}$ (KO) mice 5 days following saline or MPTP administration were analysed by western blot analyses using indicated antibodies (top panel). Quantification of the densitometric values of pT232 relative to Ape1 from western blot ($n = 3$; bottom panel). (c) Quantification of the increased densitometric values of immunofluorescence for pT232 in saline and MPTP injected $p35^{+/+}$ and $p35^{-/-}$ mice ($n = 3$). Representative images are shown in Supplementary Information, Fig. S7a. (d) Viral expression of Ape1^{WT}, Ape1^{T232A} or Ape1^{T232E}

in the SNc extracts of mice with viral injection was confirmed by western blot analyses using anti-Ape1 antibody. The upper band showed Flag-tagged Ape1 variants and the lower band showed endogenous Ape1 (C, contralateral; I, ipsilateral). Loading control is shown in the bottom panel. (e) Representative pictures of the ipsilateral side of animals injected with the indicated viruses and MPTP. Adenoviruses were injected directly into the striatum of animals for 7 days. Brains were sectioned for tyrosine hydroxylase DAB staining (N, control mouse without any treatment). Scale bar, 100 μ m. (f) Quantification of tyrosine hydroxylase (TH)-positive dopaminergic neurons from ipsilateral or contralateral sides in the indicated treatment groups ($n = 4$). (g) Quantification of cresyl violet stained neurons at the ipsilateral or contralateral sites from the indicated treatment groups ($n = 4$). In a, b, c, f and g error bars represent s.e.m. Uncropped images of the western blots are shown in Fig. S8 (a, b).

however, can also inhibit DPOLB in BER pathways²³. Accordingly, we used another AP endonuclease inhibitor 7-nitroindole-2-carboxylic acid (NAC)²⁴ which also sensitized neurons to death with or without MPP⁺ exposure (Supplementary Information, Fig. S5e). Treatment with methoxamine plus Ape1 siRNA did not additively sensitize neurons to death when compared with either treatment alone (Supplementary Information, Fig. S5f). This suggests that the mode of action of methoxamine is probably through its ability to inhibit Ape1. Finally, we examined the effects of Ape1 siRNA or roscovitine treatment in $p35^{-/-}$ neurons and showed that inhibition of Cdk5 activity partially rescued the sensitization attributable to Ape1 downregulation (Supplementary Information, Fig. S5g). These data suggest that Cdk5 also modulates other targets and are consistent with our findings that Ape1 and Prx2 both mediate death after MPP⁺ treatment, as discussed below.

Hoechst analysis (Fig. 4g) and TUNEL staining (Supplementary Information, Fig. S6a, b) showed that overexpression of Ape1^{WT} or Ape1^{T232A} with pAdtrack-CMV plasmids significantly protected neurons from MPP⁺-induced death when compared with empty plasmid (GFP) or Ape1^{T232E}. We also repeated the experiment using viral-mediated gene delivery. We generated these viruses and confirmed protein expression of adenoviral Flag-Ape1 and its mutants in HEK293 cells (Supplementary Information, Fig. S6c, top) and neurons (Supplementary Information, Fig. S6d) by western blot analyses. Viral expression of Ape1^{WT} or Ape1^{T232A} enhanced the AP endonuclease activity in HEK293 cells, whereas Ape1^{T232E} did not (Supplementary Information, Fig. S6c, bottom). The viability data from infected neurons were consistent with those of transfected neurons (Supplementary Information, Fig. S6e). These data indicate that phosphorylation of

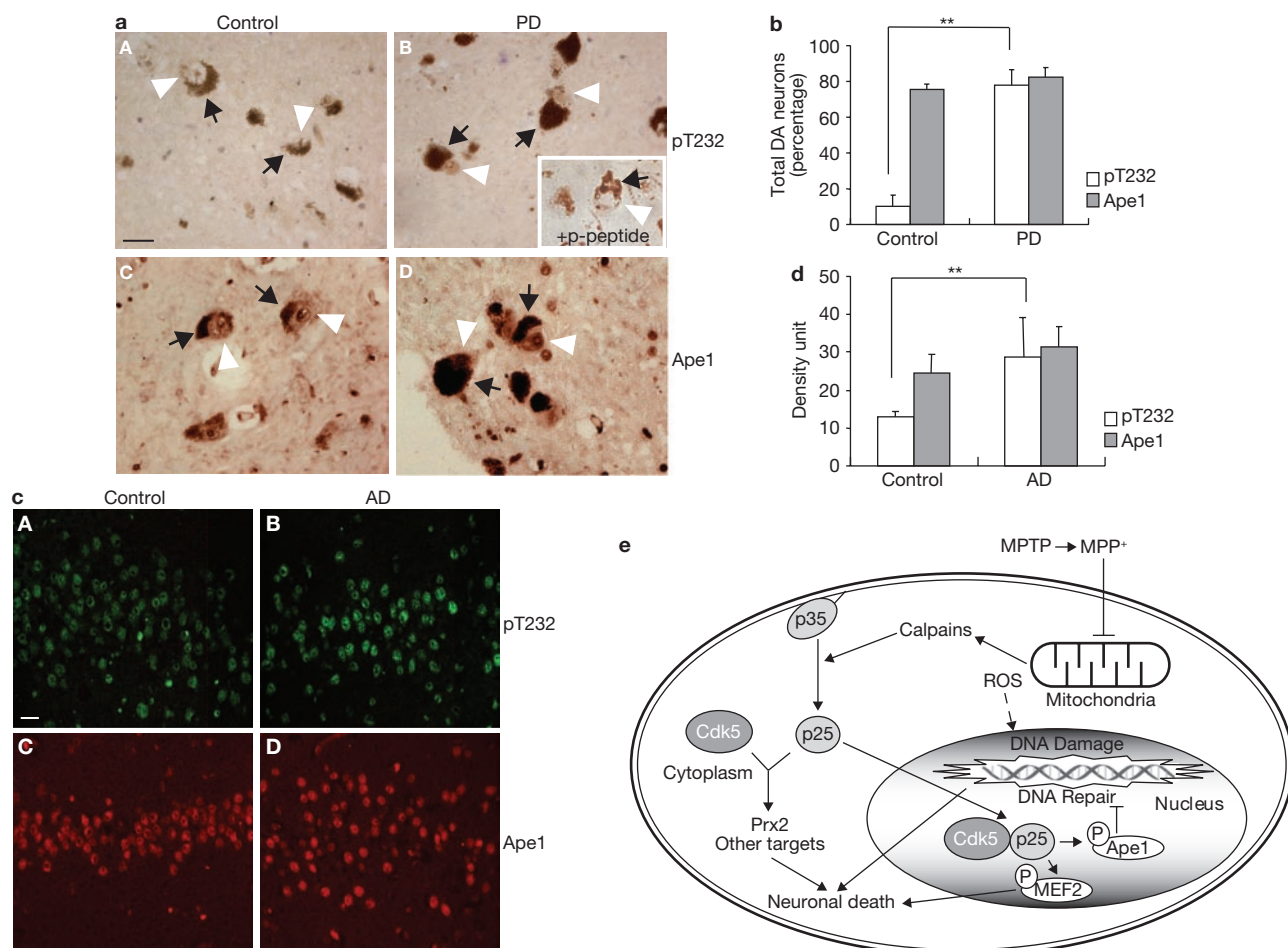


Figure 6 Ape1 phosphorylation at Thr 232 is increased in human Parkinson's disease and Alzheimer's disease. **(a)** Sections of human SNc obtained from control (A and C) or Parkinson's disease (PD; B and D) individuals were immunostained using the pT232 antibody (A and B) or anti-Ape1 antibody (C and D) and visualized by DAB staining. Neuromelanin pigment showing dopaminergic nigral neurons present as punctate brown in the cytoplasm (black arrows). Immunohistochemistry using pT232 pre-incubated with phos-peptide antigen (CNAGFpT²³²PQER; p-peptide) in Parkinson's disease sample as in B and showed that the signal of pT232 was blocked by p-peptide antigen (B inset). Scale bar, 20 μ m. **(b)** Quantification of percentage of pT232 and anti-Ape1 positive neurons to total neurons in Parkinson's disease ($n = 5$) and control ($n = 5$) individuals. Error bars represent s.e.m. **(c)** Sections of post-mortem human hippocampus obtained

from control (A and C) or Alzheimer's disease (AD; B and D) individuals were analysed by immunofluorescence staining using the pT232 antibody (A and B) and anti-total Ape1 antibody (C and D), and showed that phos-Ape1 and total Ape1 were both expressed in nuclei of pyramidal neurons in Alzheimer's disease hippocampus. Scale bar, 20 μ m. **(d)** Quantification by image analysis of density of fluorescent signal of pyramidal neurons from Alzheimer's disease ($n = 4$) and control ($n = 4$) individuals. Error bars represent s.e.m. **(e)** Model for the role of Cdk5-mediated Ape1 phosphorylation in dopaminergic neuron loss induced by MPP⁺/MPTP. Cdk5 is activated by calpain in mitochondrial stress induced by MPP⁺/MPTP. Activated Cdk5 contributes to dopaminergic loss through phosphorylation of Ape1 in the nucleus, which inactivates the AP endonuclease activity and subsequently enhances DNA damage.

Ape1 at Thr 232 accounts for the protective role of Ape1 in MPP⁺-induced neuronal death. We also confirmed that the critical role of AP endonuclease activity of Ape1 by expressing a deletion mutant of Ape1 (1–127) lacking the C-terminal portion required for AP endonuclease activity. This mutant failed to significantly protect neurons from MPP⁺ exposure (Supplementary Information, Fig. S6f). Moreover, we also examined the effect of Ape1 and its mutants on induction of AP sites that contribute to neuronal death. Infection with viruses expressing Ape1 or Ape1^{T232A}, but not GFP or Ape1^{T232E}, reduced the number of AP sites in neurons following MPP⁺ exposure (Fig. 4h). These data suggest that Ape1 protects neurons by reducing AP sites.

Our previous work indicated that Prx2 was also a critical substrate for Cdk5 (ref. 11). We therefore determined whether both Prx2 and Ape1 might act in concert to promote survival. Expression of

either protein alone gave equivalent protection. However, there was increased, additive protection when both proteins were expressed together (Fig. 4i). This suggests that these Cdk5 targets work together to promote survival. The converse results were observed when both proteins were inhibited using *Prx2* siRNA and *Ape1* siRNA treatment, or *Prx2* siRNA treatment with methoxamine (Supplementary Information, Fig. S6g, h).

Ape1 phosphorylation is induced in an MPTP mouse model

We next determined the relevance of this pathway in MPTP-injected mice. Phos-Ape1 was increased in substantia nigra pars compacta (SNc) extracts, peaking 5 days after MPTP injection (Fig. 5a). We further examined whether phosphorylation of Ape1 at Thr 232 is mediated specifically by Cdk5. The level of phos-Ape1 was markedly

reduced in $p35^{-/-}$, compared with $p35^{+/+}$ SNc extracts 5 days after MPTP administration (Fig. 5b). Consistently, immunofluorescence analyses showed that both phos-Ape1 and total Ape1 were induced and mainly expressed in nuclei of dopaminergic neurons in the SNc of $p35^{+/+}$ mice, but not in $p35^{-/-}$ mice, 5 days after MPTP administration (Fig. 5c; Supplementary Information, Fig. S7a), whereas the total Ape1 was still induced in both $p35^{+/+}$ and $p35^{-/-}$ mice (Supplementary Information, Fig. S7b, c).

Ape1 phosphorylation mimic compromises the protective role of Ape1 in an MPTP mouse model

We further examined the effects of overexpressing Ape1^{WT}, Ape1^{T232A} and Ape1^{T232E} on survival of dopaminergic neurons *in vivo* after administration of MPTP. These constructs were targeted unilaterally to dopaminergic neurons of SNc from the striatum by retrograde transport using a well established adenoviral-mediated gene delivery approach^{4,25–28}. We verified, by western blotting, protein expression of adenoviral Ape1 or its mutants in SNc of mice (Fig. 5d) and immunofluorescence (Supplementary Information, Fig. S7d). Overexpression of Ape1^{WT} or Ape1^{T232A} resulted in significant dopaminergic neuroprotection after administration of MPTP when compared with the contralateral untreated side, or to the GFP-injected control animals, as analysed by counting the number of neurons positive for tyrosine hydroxylase (Fig. 5e, f). In contrast, expression of Ape1^{T232E} showed effects that were similar to the GFP controls (Fig. 5e, f). Cresyl violet staining showed similar results to those of tyrosine hydroxylase assessment (Fig. 5g). These data indicate that Ape1 protects dopaminergic neurons in an MPTP mouse model, and mimicking Ape1 phosphorylation at Thr 232 compromises the protection.

Relevance to human Parkinson's disease and Alzheimer's disease

We further examined the potential relevance of Ape1 phosphorylation to human disease conditions. Obvious immunoreactivity of phos-Ape1 was observed in the nuclei of dopaminergic neurons from post-mortem tissue of patients with Parkinson's disease (white arrowheads, Fig. 6aB), whereas little or no signal was detected in the normal control tissues (white arrowheads, Fig. 6aA). A significant increase in the proportion of phos-Ape1-positive to total dopaminergic neurons was observed in tissue from patients with Parkinson's disease when compared with normal control tissue (Fig. 6b). The proportion of dopaminergic neurons positive for total Ape1 did not differ between normal tissue (Fig. 6aC) and tissue from patients with Parkinson's disease (Fig. 6aD, b). We also examined brains from patients with Alzheimer's disease and showed that the density of the phos-Ape1 signal, but not total Ape1 signal, was significantly elevated in the nucleus of pyramidal neurons in the hippocampus of Alzheimer's disease patients (Fig. 6c, d; Supplementary Information, Fig. S7e). The signal of pT232 was blocked by pre-incubation with p-peptide antigen in samples from patients with Parkinson's disease (Fig. 6aB inset) and Alzheimer's disease (Supplementary Information, Fig. S7e), supporting the specificity of pT232 antibody.

DISCUSSION

DNA damage and defects in DNA repair have been linked to neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease^{29,30}. Here we have discovered a signalling link between the Cdk5 pathway and DNA damage/DNA repair defects in neuronal death by the identification of a previously unknown Cdk5 target, Ape1. We have

demonstrated that p35 interacts directly with Ape1, and that both Cdk5/p35 and Cdk5/p25 complexes can phosphorylate Ape1 at Thr 232, leading to reduced AP endonuclease activity. Moreover, we have demonstrated that endogenous Ape1 is phosphorylated at this site, *in vitro* and *in vivo*, after exposure to MPP⁺/MPTP, and that this is dependent on Cdk5. Inhibition of Ape1 phosphorylation not only increased the AP endonuclease activity but also reduced the presence of AP sites resulting from exposure to toxins. Substantial evidence indicates that accumulation of AP sites contributes to cell death^{17,31}, although it may not be the only contributor. This is consistent with recent reports that Ape1-deficient cells are associated with accumulation of cytotoxic, unrepaired AP sites^{32,33}. In addition, overexpression of Ape1^{WT} or Ape1^{T232A}, but not Ape1^{T232E}, has a protective role in neuronal death *in vitro* and *in vivo*. However, it is unclear whether Ape1 acts solely through AP site repair and whether it is possible that other factors may also contribute to MPP⁺/MPTP-induced death. Nonetheless, these observations support the notion that Cdk5-mediated phosphorylation of Ape1 at Thr 232 is a critical factor promoting neuronal death. It is also consistent with increasing reports of links between Cdk5 and DNA damage^{7,34–36}.

Our previous and present work clearly demonstrate the importance of Cdk5 in models of dopaminergic neuron loss^{4,11}. However, it is also clear that Cdk5 probably acts through multiple targets, including but not limited to Ape1. For example, we have previously shown that Cdk5 can phosphorylate and inactivate other targets important for neuronal survival: an antioxidant enzyme Prx2 (ref. 11) and transcription factor MEF2 (refs 3, 5). We propose that coordinated targeting of multiple Cdk5 targets contributes to stress-induced neuronal death (see Fig. 6e).

Several studies have indicated that hyperactive Cdk5 is mainly maintained by the cleavage of p35 to p25 mediated by the calcium-dependent proteases calpains^{37,38}. Mitochondrial dysfunction triggered by MPP⁺ can evoke a sustained elevation of cytoplasmic calcium levels³⁹, which could activate calpains. Consistent with our previous studies^{3,11,12}, the present data have shown that inhibition of calpains can block the induction of Ape1 phosphorylation at Thr 232 in MPP⁺-treated neurons, supporting the involvement of calpains in Cdk5 activation. The distinction between p25 and p35 and the deregulated nature of p25 are interesting from another perspective. We have shown that Cdk5 complexes containing p25 or p35 can phosphorylate Ape1. Yet, only p35/Cdk5 complexes can stably bind Ape1. Clearly, stable binding by kinases is not required for phosphorylation of substrate^{40,41}. We have observed that p10 expression interferes with the ability of complexes containing p35, but not p25, to phosphorylate Ape1. This suggests that the p35 interaction through its p10 fragment has a regulatory role that is absent in p25. This observation also reinforces the deregulated nature of p25.

Finally, consistent with the data from our *in vitro* and *in vivo* models of neuronal stress, we have also provided evidence that Cdk5-mediated Ape1 phosphorylation at Thr 232 is elevated in post-mortem brain samples from patients with Parkinson's disease and Alzheimer's disease. These findings are consistent with several reports implicating Cdk5 involved in Parkinson's disease⁴² and Alzheimer's disease^{8,43}. With all the relevant caveats of post-mortem analyses, these results provide a potential tantalizing link to human neurodegenerative conditions. In summary, the present study defines a critical Cdk5-mediated DNA repair pathway and a potential for development of a unique therapeutic strategy for neurodegenerative conditions. □

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturecellbiology/>

Note: Supplementary Information is available on the Nature Cell Biology website.

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AUTHOR CONTRIBUTIONS

E.H., Q.D., R.S.S., D.S.P. designed the studies; E.H. and D.Q. performed most of the experimental work; Y.Z., K.V., M. E. H. and M.W.C.R. provided technical support; J. M. W. provided post-mortem Parkinson's disease and Alzheimer's disease samples and analyses; E.H., Q.D. and D.S.P. analysed data and wrote the manuscript; D.S.P. supervised the project.

COMPETING INTERESTS

The authors declare no competing financial interests.

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METHODS

Antibodies. The following antibodies were used: tyrosine hydroxylase (TH; 1:10,000; Immunostar), C-8 for Cdk5 (1:5,000; Santa Cruz), C-19 for p35 (1:5,000; Santa Cruz), β -actin (1:50,000; monoclonal; Sigma), Ape1/Ref-1 (1:5,000; Santa Cruz), Cleaved caspase-3 (Asp175) (1:100; Cell Signaling), Ogg1 (1:10,000; NB100-106, Novus Biologicals), DPOLB (1:2,500; NB100-91734; Novus Biologicals), Phos-Ape1 at Thr 232 (pT232, 1:4,000) polyclonal antibody was generated and purified from a rabbit immunized with carrier protein-conjugated phosphopeptide, CNAGFT²³²PQER (residues 228–236 of mouse Ape1), using standard protocols by Biogenes.

Plasmid construction. The coding region of Ape1 was amplified by RT-PCR from mouse brain mRNA and cloned into pQE-30 vector (His-tag, Qiagen). The sequence of Ape1 was verified by DNA sequencing. Ape1 mutants (T232A, T232E, S53A, S53E and S53AT232A (ST2A)) were generated using QuickChange site-directed mutagenesis kit (Stratagene) in the pQE-30 vector (His-tag, Qiagen). After an N-terminal 3XFlag tag was introduced, the Flag-Ape1 variants without His-tag were subcloned into the pAdtrack-CMV vector for preparation of recombinant adenovirus as described previously⁴⁴. The viral constructs expressed Flag-Ape1 variants and GFP separately because they are controlled by separate CMV promoters. Wild-type Cdk5 and p25 cDNA were engineered into the pGEX2T vector for preparation of GST fusion proteins⁴⁵.

Fusion proteins, *in vitro* binding assay and kinase assay. All GST- or His-tagged fusion proteins were expressed in *Escherichia coli* and were purified using Sepharose 4B agarose (GE Healthcare) or Ni-NTA Agarose (Qiagen) according to the manufacturer's instruction. *In vitro* binding assay and Cdk5 kinase assay was performed as described previously⁴⁵.

Immunoprecipitation. Samples were collected 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 were performed through incubation of antibodies with lysates overnight followed by incubation with protein A beads (Sigma, USA) at 4°C for 2 h.

AP endonuclease activity assay. The AP-specific endonuclease activity of Ape1 was quantified by measuring incision of a HEX-5'-end-labelled 26-mer duplex oligonucleotide substrate containing a synthetic tetrahydrofuran (THF, F) abasic site (5'-AATTCACCGGTACCFTCTAGAATTCG-3'; GeneLink) as described previously⁴⁶. The abasic analogue is resistant to cleavage by 3'-acting AP lyase activity, which is generally possessed by DNA glycosylases/AP lyases. Therefore, this assay is specific for Ape1 activity⁴⁷. Recombinant protein or cell extract was quantified using the Bradford assay. Reaction mixtures (20 μ l) containing 10 ng Ape1 variant fusion protein or 100 ng cell extract, 5 pmol of HEX-5' end-labelled, double-stranded THF oligonucleotide, 50 mM HEPES, 50 mM KCl, 10 mM MgCl₂, 1 μ g ml⁻¹ BSA, and 0.05% Triton X-100 (pH 7.5) were incubated for 15 min in a 37°C water bath. The reactions were then stopped by adding 20 μ l formamide and 10 mM EDTA. The reaction mixtures were separated by a 20% polyacrylamide gel containing 7 M urea. The bands of 14-mer (product) and 26-mer (substrate) were detected using the AlphaImager 2200 Imaging System and the densitometric values of the bands were quantified. The AP endonuclease activity of each reaction was calculated as densitometric value of ((product/(product + substrate)) $\times$ 100.

Cortical neuronal cultures, transfection, survival assay and RNA interference. Primary culture of mouse cortical neurons were carried out as described previously^{48,49}. For transfection, 72 h after initial plating, the cortical neurons were transiently transfected using the Lipofectamine 2000 (Invitrogen) protocol as described previously⁵⁰. For co-transfection, 3 μ g of plasmid DNA without GFP was transfected with 1 μ g reporter plasmids containing GFP (that is, pAdtrack or pEGFP) in 24-well neurons. 24 h after transfection, cortical neurons were exposed to 20 μ M MPP⁺ for 48 h and subjected to survival determination.

Survival was determined as described previously⁵¹, by incubating neurons in a buffer that selectively lyses cell membranes but not nuclear membranes and then evaluating the number of healthy intact nuclei. The percentage of surviving neurons is expressed relative to untreated control wells. For survival of transfected neurons, GFP positive neurons were counted as either alive or dead according to

the appearance of Hoechst 33258 (0.5 ng ml⁻¹) staining. Survival was expressed as the percentage of alive to total cells. Alternatively, TUNEL staining was performed as described previously⁵².

For RNA interference studies, neurons were transfected with 60 pmol/24-well (24-well plate) siRNA to mouse Ape1 (S1: sc-36401, Santa Cruz; S2: 65892, Ambion) or control siRNA (S1: sc-37007, Santa Cruz; S2: 4390843, Ambion), or co-transfected with siRNA Ape1-S2 (30 pmol/24-well) and siRNA-Prx2 (187225, Ambion, 30 pmol/24-well) using Lipofectamine 2000 (Invitrogen) as described previously⁵¹. Twenty-four hours after transfection with siRNA, neurons were treated with 20 μ M MPP⁺ or saline for 48 h and then processed for survival assay. We have observed that targeting of duplexes to neurons is much more efficient than that of plasmids using this procedure previously^{51,53}. Ape1-S1 siRNA from Santa Cruz consists of multiple siRNAs and is provided as pools of 3 targets specific for 20–25 nucleotides of siRNAs designed to specifically knock down the expression of mouse Ape1. Ape1-S2 siRNA (5'-GGAAACAAAAAGAAUGCUG) from Ambion targets mouse 736–754 bp of Ape1 mRNA and the targeted consensus sequence in human Ape1 mRNA is 673–691 bp (5'-GGGAACAAAAAGAATGCTG), where the underlined third 'G' is different from mouse base 'A'.

Detection of apurinic/aprimidinic (AP) sites. ARP reagent (N'-aminooxy methylcarbonylhydrazino-D-biotin; DN-002; Kamiya Biomedical Company) reacts specifically with an aldehyde group, which is the open ring form of the AP sites. After treating DNA containing AP sites with ARP reagent, AP sites are tagged with biotin residues and can be quantified using avidin-biotin assay followed by a colorimetric detection of peroxidase conjugated to the avidin. Cellular genomic DNA was isolated with a phenol/chloroform/isoamyl alcohol solution (Invitrogen). 1 μ g DNA was subjected to an AP sites quantification assay, according to the manufacturer's instructions, as described previously^{32,33}. Data were presented as the number of AP sites per 10⁵ nucleotides, as determined against a standard supplied by the manufacturer.

Animals and MPTP administration. Eight-week-old male C57BL/6 mice (22–28 g; Charles River Laboratories, Canada) were used for MPTP experiments. p35-deficient mice (maintained on a C57BL/6 background) and wild-type littermates were generated by breeding their heterozygote p35 counterparts. Genotyping information was reported previously²¹. 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 with approval from the University of Ottawa Animal Care Committee.

Mice received one intraperitoneal injection of MPTP-HCl per day (30 mg kg⁻¹, Sigma) for five consecutive days^{4,26–28}. Control mice received an equivalent volume of 0.9% saline. Brains were extracted at indicated times or perfused for immunohistochemical analyses.

Intrastriatal administration of adenoviruses. The adenoviruses expressing GFP, Ape1^{WT}, Ape1^{T232A} or Ape1^{T232E} were engineered using pAdEasy system as described previously^{44,54}. We have previously shown that adenoviruses can target the SNc from the striatum by retrograde transport^{4,25–28}. A single unilateral injection of each virus (2 μ l, 1 $\times$ 10⁷ particles per μ l) was delivered to the right striatum (0.5 mm rostral, 2.2 mm right of bregma, and 3.4 mm below the skull surface) of animals using a syringe pump system 7 days before MPTP administration. Brains were extracted for immunohistochemistry 14 days after the first MPTP treatment. To examine the viral expression in TH neurons, a double-labelling immunofluorescence was performed using specific primary antibody to GFP (Abcam) or tyrosine hydroxylase (Immunostar) 7 days after virus injection.

Immunohistochemistry and immunofluorescence. Mice were perfused transcardially, and brains were fixed in 4% paraformaldehyde and cryoprotected as described previously²⁵. Free-floating serial coronal sections (14 μ m thickness) of the ventral midbrain were collected. Sections were then incubated with tyrosine hydroxylase antibody (1:10,000) for 24 h at 4°C. Immunoreactivity was visualized using an avidin-biotin complex peroxidase/3, 3'-diaminobenzidine (DAB) reaction. For double-labelling immunofluorescence, samples were double-labelled with pT232 (rabbit) with mouse anti-MAP2 (Santa Cruz), and anti-Ape1 (mouse) antibody with rabbit anti-MAP2 (Santa Cruz), and visualized using either Alex-488-conjugated anti-rabbit IgG (1:200) or Alex-594-conjugated anti-mouse IgG (1:200).

Quantification of dopaminergic neuron loss. The number of dopaminergic (positive for tyrosine hydroxylase) neurons was only counted from the sections in the region containing the medial terminal nucleus because this region has been previously shown to express the highest level of virus-mediated gene expression after intrastriatal infection²⁶. We also used the medial terminal nucleus as a landmark to evaluate consistent levels of the substantia nigra compact (SNc). Neurons in the ipsilateral and contralateral side to the viral injection were assessed as described previously²⁶. At least three sections per animal were analysed. Alternatively, cresyl violet staining was performed to validate determination of nigral counts as described previously²⁶.

Human brain samples. Paraffin-embedded blocks of post-mortem human Parkinson's disease and Alzheimer's disease samples 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. Diagnoses of Parkinson's disease and Alzheimer's disease were based on medical histories and post-mortem confirmation (JMW). The tissue was deparaffinized in xylene and subjected to citrate antigen retrieval⁵⁵ before DAB or immunofluorescence staining. The average age and postmortem intervals for Parkinson's disease ($n = 5$; 3 males and 2 females) and control patients ($n = 5$; 5 males) were not significantly different ($P > 0.60$ and $P > 0.30$, respectively). The average age and post-mortem intervals for Alzheimer's disease ($n = 4$; 2 males and 2 females) and control patients ($n = 4$; 1 male and 3 females) were not significantly different ($P > 0.20$ and $P > 0.10$, respectively).

Statistical analysis. Differences between two groups of data were analysed with two-tailed Student's t -test. The data are presented as mean $\pm$ s.e.m.; * $P < 0.05$; ** $P < 0.01$ denotes statistical significance.

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Loss of the Parkinson's disease-linked gene DJ-1 perturbs mitochondrial dynamics

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Growing evidence highlights a role for mitochondrial dysfunction and oxidative stress as underlying contributors to Parkinson's disease (PD) pathogenesis. DJ-1 (PARK7) is a recently identified recessive familial PD gene. Its loss leads to increased susceptibility of neurons to oxidative stress and death. However, its mechanism of action is not fully understood. Presently, we report that DJ-1 deficiency in cell lines, cultured neurons, mouse brain and lymphoblast cells derived from DJ-1 patients display aberrant mitochondrial morphology. We also show that these DJ-1-dependent mitochondrial defects contribute to oxidative stress-induced sensitivity to cell death since reversal of this fragmented mitochondrial phenotype abrogates neuronal cell death. Reactive oxygen species (ROS) appear to play a critical role in the observed defects, as ROS scavengers rescue the phenotype and mitochondria isolated from DJ-1 deficient animals produce more ROS compared with control. Importantly, the aberrant mitochondrial phenotype can be rescued by the expression of Pink1 and Parkin, two PD-linked genes involved in regulating mitochondrial dynamics and quality control. Finally, we show that DJ-1 deficiency leads to altered autophagy in murine and human cells. Our findings define a mechanism by which the DJ-1-dependent mitochondrial defects contribute to the increased sensitivity to oxidative stress-induced cell death that has been previously reported.

INTRODUCTION

Parkinson's disease (PD), the second most common neurodegenerative disorder, is characterized by the progressive loss of neurons within the substantia nigra *pars compacta* (1,2). Though the pathogenic mechanisms underlying PD are not well understood, growing evidence supports a role for mitochondrial dysfunction, oxidative stress and more recently autophagy.

Mitochondrial dysfunction was initially tied to PD in studies demonstrating the presence of aberrant mitochondrial function in idiopathic PD patients (3,4). Moreover, several dopamin-

ergic toxins acted as mitochondrial toxins by inhibiting the electron transport chain, producing toxic-free radicals in the process (5,6). Since this time, several familial PD genes, including, Parkin (PARK2), Pink1 (PARK6) and DJ-1 (PARK7), have been linked to mitochondria. Their loss results in abnormal mitochondrial morphology (7,8). Interestingly, the interplay of Pink1 and Parkin dynamically regulates mitochondrial morphology via mitochondrial fission/fusion and also affects mitochondrial quality control (9–11). As the function of Pink1 and Parkin in these contexts continues to be elucidated, the role(s) of DJ-1 is less understood.

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Homozygous loss-of-function mutations in DJ-1 (PARK7) result in early onset PD (12). Several lines of evidence, including our own, indicate that DJ-1 protects neurons against oxidative stress-induced cell death (13,14). It has been postulated that DJ-1 exerts its protective function by regulating mitochondrial homeostasis or participating in the oxidative stress response either serving as an antioxidant scavenger or a redox sensor (14–17). More recently, DJ-1 was found to affect mitochondrial quality control (18,19). Given the importance of reactive oxygen species (ROS) in regulating mitochondrial dynamics and the observations that loss of Pink1 and Parkin has also been linked to mitochondrial dysfunction, we wanted to address whether DJ-1 also affects mitochondrial dynamics and function.

Here we examined mitochondrial morphology and function in DJ-1 deficient tissues and hypothesized that loss of DJ-1 would produce a fragmented mitochondrial phenotype, accounting increased sensitivity to cell death of DJ-1 deficient neurons previously reported (14). We demonstrate that DJ-1 deficiency leads to a fragmented mitochondrial phenotype in multiple contexts including neurons and human DJ-1 patient cells. Second, we provide evidence that ROS plays a critical role in this fragmentation phenotype and that DJ-1 deficiency results in elevated ROS levels. Third, we show that this mitochondrial phenotype is an important contributor to the sensitivity to oxidative stress caused by the loss of DJ-1. Fourth, we show that Pink1 and Parkin can rescue the mitochondrial fragmentation induced by the loss of DJ-1. Finally, we also show that the loss of DJ-1 results in increased autophagic activity.

RESULTS

Loss of DJ-1 alters mitochondrial morphology and dynamics

Based upon the growing evidence for mitochondrial morphology and dynamics as underlying contributors to PD, we first investigated a role for DJ-1 in mitochondrial remodeling. Primary cortical neurons and mouse embryonic fibroblasts (MEFs) from DJ-1^{+/+} or DJ-1^{-/-} embryos (E15.5) were cultured. Mitochondria were quantified and binned according to length, as done previously (20). As shown in Figure 1A and quantified in Figure 1B, mitochondrial lengths in DJ-1^{+/+} primary cortical neurons at 3 days *in vitro* were significantly longer and less fragmented than in DJ-1^{-/-} neurons. This fragmented mitochondrial phenotype was also evident in MEFs (Fig. 1C and D) and *in vivo* in the striatum of DJ-1^{+/+} and DJ-1^{-/-} mice (Fig. 1E and F). Thus, the fragmented morphology appears to be a more generalized phenomenon rather than restricted to a specific cell type, occurring both *in vitro* and *in vivo*. These data demonstrate that mitochondrial morphology is altered with the loss of DJ-1.

To address whether the DJ-1-dependent mitochondrial fragmentation was related to alterations in mitochondrial fusion rates, DJ-1^{+/+} or DJ-1^{-/-} MEFs were transduced with a matrix-targeted photoactivatable GFP lentivirus (PA-GFP). PA-GFP was activated in ~10% of the cell using a 405 nm laser line at 75% intensity (21). Upon photoactivation, the spread of the GFP signal throughout the mitochondrial reticu-

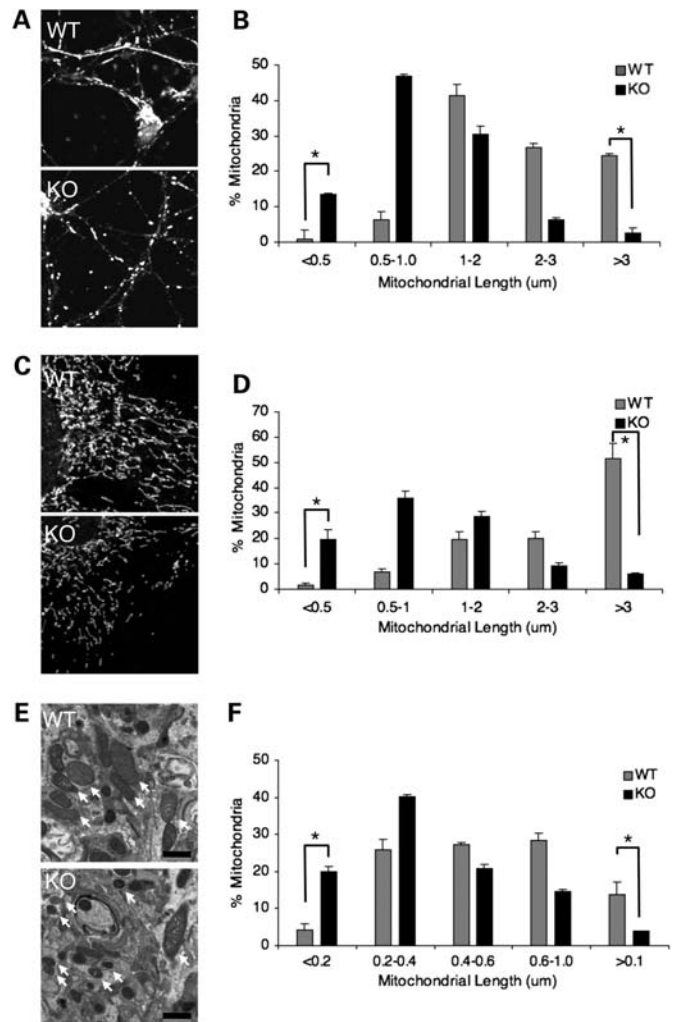


Figure 1. DJ-1 deficiency results in altered mitochondrial morphology *in vitro* and *in vivo*. (A) Primary cortical neurons (3 DIV) and (C) MEFs from wild-type (WT) and knockout (KO) DJ-1 embryos were harvested and fixed as described under Materials and Methods and immunostained with antibodies to Tom20 to visualize mitochondria. Quantification of mitochondrial lengths in (B) primary cortical neurons and (D) MEFs was done as described previously [Jahani-Asl *et al.* (20); $n=4$ independent experiments with a minimum of 500 mitochondria/experiment counted]. Scale Bar = 2 μ m. * $P < 0.05$ versus respective $+/+$ control. (E) Electron microscopic images of WT and KO DJ-1 striatum prepared as described in Materials and Methods. (F) Quantification of mitochondrial diameters in the striatum of WT and KO DJ-1 mice from three mice/genotype. Scale bar = 500 nm. * $P < 0.05$ versus respective WT DJ-1 control. DIV, days *in vitro*. White arrows in (E) depict mitochondria.

lum was assessed immediately post-activation and following 20 min (Fig. 2A). The data in Figure 2B demonstrate that mitochondrial fusion in MEFs is decreased by 30% DJ-1^{-/-} when compared with DJ-1^{+/+}. Steady-state levels of the mitochondrial fission and fusion proteins, Dynamin Related Protein-1 (Drp1) and mitofusin 1 (MFN1) were also measured to determine whether the loss of DJ-1 would result in altered expression. As shown in Figure 2C, Drp1 levels were not altered, while decreases in the levels of MFN1 were observed.

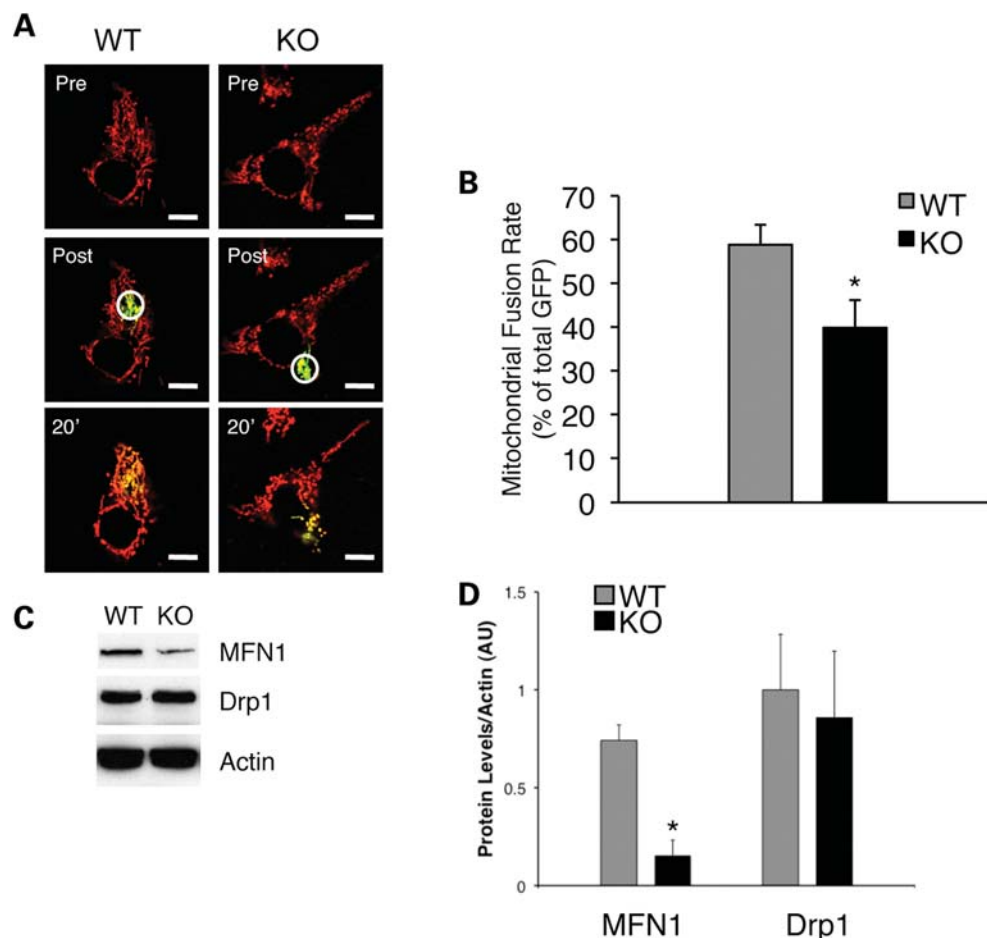


Figure 2. Mitochondrial fusion is decreased with DJ-1 deficiency. (A) Confocal images of mitochondria from DJ-1^{+/+} (WT) and DJ-1^{-/-} (KO) MEFs transduced with mitochondrial matrix-targeted DS-red and PA-GFP lentiviruses as described in the Supplementary Information. Images shown are from pre-activation (Pre), immediately following activation (Post) in a small region of interest (indicated by open white circles) as well as following 20 min (20') of activation. (B) Quantification of mitochondrial fusion 20 min post-activation from DJ-1^{+/+} ($n = 14$ cells) and DJ-1^{-/-} ($n = 12$ cells) MEFs. (C) Protein extracts were made from DJ-1^{+/+} and DJ-1^{-/-} MEFs and subjected to western blotting for Drp1, Mfn1 and actin (for loading control). Data shown are representative of at least three independent experiments. (D) Quantification of Drp1 and MFN1 protein levels, corrected with actin for loading in DJ-1 WT and KO MEFs. * $P < 0.05$.

Rescue of mitochondrial length in DJ-1^{-/-} neurons abrogates neuronal cell death

Our previous work has shown that overexpression of DJ-1 protects primary cortical neurons from oxidative stress (14). Here we report that DJ-1 deficiency promotes mitochondrial fragmentation. To determine whether these phenomena are linked, primary cortical neurons from DJ-1^{+/+} and DJ-1^{-/-} embryos were infected with dominant-negative dynamin-related protein 1 (DRP1K38E), a mutant form of the mitochondrial fission factor that promotes an elongated mitochondrial reticulum when expressed in cells. Expression levels of Drp1 K38E are shown in Figure 3A and were previously described (21). When primary cortical neurons were subjected to oxidative stress in the form of MPP⁺ (10 μ M), a metabolite of the parkinsonism-inducing drug MPTP (22) for 48 h, the hypersensitive DJ-1^{-/-} neurons showed an increase in cell death. However, DJ-1^{-/-} cortical neurons infected with DRP1K38E were completely protected from the toxic effects of MPP⁺ suggesting that mitochondrial fragmentation contributes to oxidative stress-induced sensitivity to cell death (Fig. 3B).

NAC treatment rescues the mitochondrial phenotype in DJ-1^{-/-} neurons

ROS can significantly influence mitochondrial morphology, producing a fragmented phenotype (23). Thus, to assess whether the DJ-1-dependent mitochondrial morphology is related to ROS, we determined whether quenching with N-acetyl-L-cysteine (NAC) might affect mitochondrial fragmentation observed in DJ-1 deficient cells. DJ-1^{+/+} and DJ-1^{-/-} primary cortical neurons were incubated with the ROS scavenger NAC (1 mM) for 48 h (Fig. 4A). Quantification of mitochondrial lengths in vehicle-treated (VEH) DJ-1^{+/+} and DJ-1^{-/-} neurons revealed a similar pattern of mitochondrial morphology deficits as described in Figure 1. While treatment with NAC did not significantly alter mitochondrial length in the DJ-1^{+/+} neurons, treatment of DJ-1^{-/-} neurons with NAC completely reversed the mitochondrial fragmentation where the percentage of mitochondria exhibiting lengths greater than 3 μ m increased (i.e. $1.14 \pm 0.305\%$ in KO-VEH to $32.144 \pm 3.141\%$ in KO-NAC; Fig. 2B) and the percentage of fragmented mitochondria decreased (i.e.

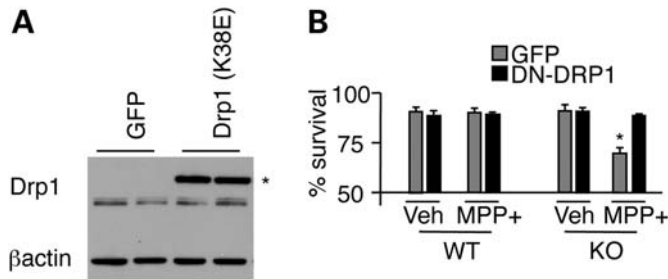


Figure 3. Mitochondrial length is critical for neuronal cell survival. (A) Primary cortical neurons infected with either GFP or DN-Drp1 were treated with vehicle (Veh) or MPP+ (10 μ M) for 24 h. (B) Cell survival was assessed by counting infected cells with intact or dead nuclei plotting the ratio of live:dead cells in treated and untreated DJ-1^{+/+} and DJ-1^{-/-} ($n = 3$ independent experiments, each experiment was performed in triplicate).

<0.5 μ m; $15.928 \pm 3.03\%$ in KO-VEH versus $0.198 \pm 0.038\%$ in KO-NAC; Fig. 4B), suggesting that elevated levels of ROS can cause mitochondrial fragmentation, which can be reversed if ROS levels are reduced.

Wild-type DJ-1 but not the DJ-1 C106A mutant rescue mitochondrial morphology defects

It has been previously reported that DJ-1 exerts its effect on oxidative stress via an isoelectric pH shift resulting in a more acidic molecule (24). Importantly, the residue that appears to be sensitive to oxidative modification, in particular hydrogen peroxide-induced oxidation, is a cysteine residue in position 106 (25,26). Thus, to provide additional relevance for the role of ROS and the importance of DJ-1 and oxidative stress in the regulation of mitochondrial morphology, we investigated whether DJ-1 itself actively regulates mitochondrial morphology and to further ascertain whether the DJ-1 mutant that is defective in handling ROS would fail to rescue the DJ-1 deficient phenotype. DJ-1^{+/+} and DJ-1^{-/-} cortical neurons were infected with adenoviruses encoding GFP (as a control), wild-type DJ-1 (DJ-1) or an oxidant mutant form of DJ-1 (C106A). This DJ-1 mutant harbors a cysteine to alanine point mutation at amino acid 106 rendering the oxidative capacity of DJ-1 non-functional. Expression levels of viruses are shown in Supplementary Material, Fig. S1. As shown in Figure 5A and quantified in Figure 5B, DJ-1^{+/+} or DJ-1^{-/-} cortical neurons infected with GFP virus alone display the wild-type mitochondrial phenotype as shown in Figure 1, demonstrating that viral expression of GFP alone does not significantly alter mitochondrial length. Next, while overexpression of DJ-1 had no effect on mitochondrial morphology in DJ-1^{+/+} neurons, DJ-1 expression in DJ-1^{-/-} neurons increased the percentage of mitochondria exhibiting lengths greater than 3 μ m ($5.15 \pm 0.826\%$ in KO-GFP versus $47.97 \pm 12.51\%$ in KO DJ-1) and decreased the percentage of fragmented mitochondria ($42.00 \pm 2.56\%$ in KO-GFP versus $1.403 \pm 1.4\%$ in KO DJ-1) supporting the idea that wild-type DJ-1 plays a role in regulating mitochondrial morphology. On the other hand, the oxidant mutant C106A cannot recapitulate the full rescue displayed by WT DJ-1 indicating that the redox function of DJ-1 is critical in promoting a fused mitochondrial reticulum.

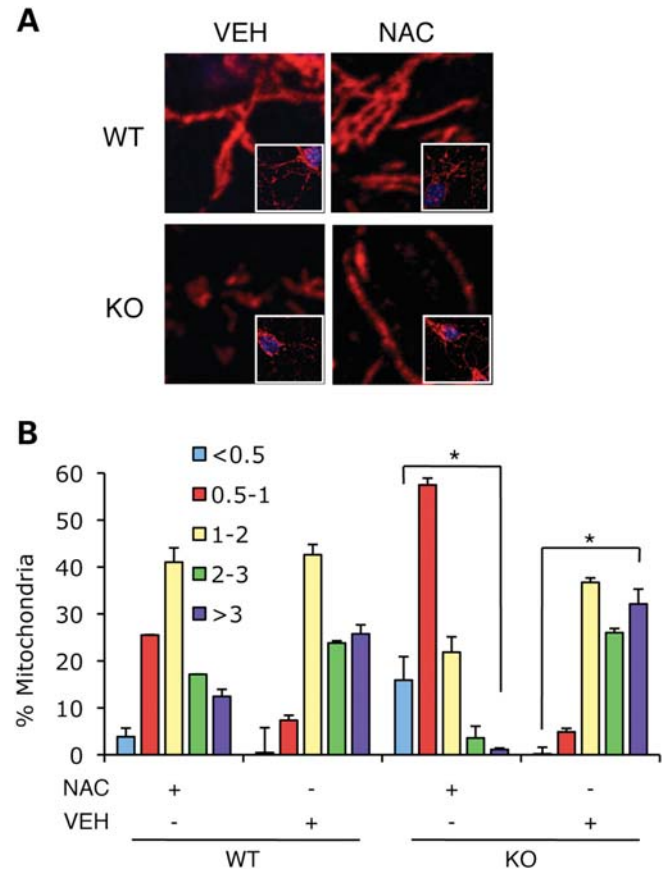


Figure 4. NAC rescues the mitochondrial morphology in DJ-1-deficient primary cortical neurons. (A) Confocal images of neurons taken from vehicle- (VEH) and NAC-treated (NAC) WT and KO neurons. Neurons were harvested and fixed 48 h post-treatment and immunostained with antibodies to Tom20 (red) to visualize mitochondria. Scale bar = 2 μ m. Inset: lower magnification images. (B) Quantification of mitochondrial lengths as described previously [Jahani-Asl *et al.* (20); $n = 3$ independent experiments with a minimum of 500 mitochondria/experiment were counted]. Scale bar = 2 μ m. * $P < 0.05$ versus respective controls.

DJ-1 deficiency alters ROS production

If ROS were indeed important in promoting the fragmented mitochondrial phenotype induced by DJ-1 deficiency, we would expect that ROS production would be elevated in mitochondria isolated from DJ-1^{-/-} mice when compared with DJ-1^{+/+} controls. Accordingly, we isolated mitochondrial fractions from brain and skeletal muscle; tissues typically associated with high metabolic requirements and mitochondria and are therefore significant sources of ROS. As predicted, we observed that H₂O₂ production in mitochondria isolated from DJ-1^{-/-} mice is increased 1.4-fold ($P < 0.05$) compared with DJ-1^{+/+} controls in both brain (Fig. 6) and skeletal muscle (Supplementary Material, Fig. S2A), respectively. In either tissue, H₂O₂ production in the DJ-1^{-/-} animals was not further increased with the addition of the mitochondrial Complex I inhibitor rotenone, suggesting that ROS production in DJ-1 deficient mitochondria is generated primarily via Complex I. Despite the increased H₂O₂ production, we did not observe gross differences in mitochondrial function

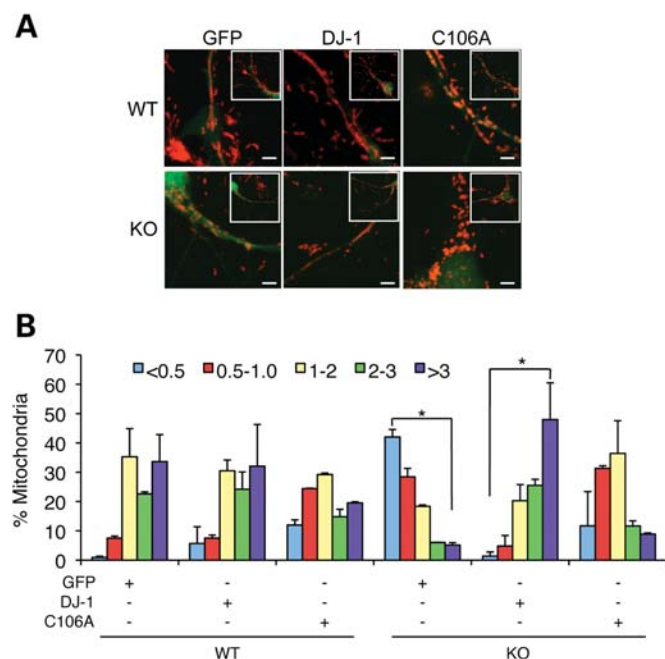


Figure 5. Restoration of wild-type mitochondrial phenotype upon re-expression of DJ-1 *in vitro*. (A) Confocal images from WT and KO DJ-1 primary cortical neurons infected with GFP, GFP-DJ-1 and GFP-DJ-1 C106A adenoviruses as described in Materials and Methods. Neurons were harvested and fixed 48 h post-infection and immunostained with antibodies to Tom20 (red) to visualize mitochondria. Inset: lower magnification images. (B) Quantification of mitochondrial lengths as described previously [Jahani-Asl *et al.* (20); $n = 3$ independent experiments with a minimum of 500 mitochondria/experiment that were counted per condition]. * $P < 0.05$ versus respective controls.

measurements that were performed such as mitochondrial respiration and citrate synthase activity in DJ-1^{-/-} mice, at least in the brain (Supplementary Material, Fig. S2B and S2C). However, it should be noted that both mitochondrial respiration and citrate synthase activity were decreased in skeletal muscle (Supplementary Material, Fig. S2D and S2E).

Pink1 and Parkin rescue mitochondrial length in DJ-1^{-/-} primary cortical neurons

Previous work conducted in *Drosophila* has demonstrated that Pink1 and Parkin participate in mitochondrial remodeling and are part of the same genetic pathway where Pink1 is upstream of Parkin (9–11,27–29). More recent evidence in mammalian cells is supportive of this notion and also implicates Parkin and Pink1 in the regulation of autophagy, a lysosomal degradation pathway responsible for the degradation of damaged proteins and organelles, including mitochondria (29–31). Thus, we determined whether Pink1 and Parkin could rescue the mitochondrial phenotype in DJ-1^{-/-} primary cortical neurons. Accordingly, we infected DJ-1^{+/+} and DJ-1^{-/-} primary cortical neurons with Pink1 and Parkin viruses, and quantified mitochondrial length as before. Viral expression of Parkin was confirmed in Supplementary Material, Fig. S3.

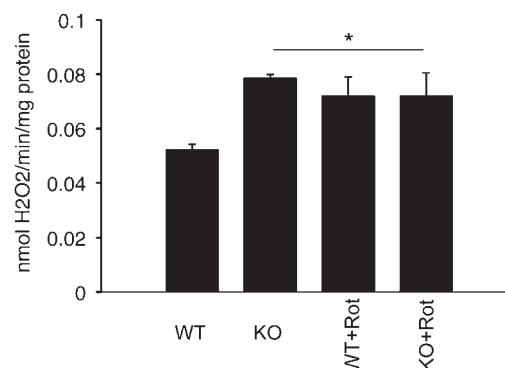


Figure 6. Brain mitochondria isolated from DJ-1 deficient animals produce more ROS. H₂O₂ production was measured in mitochondria isolated from WT and KO DJ-1 brains. * $P < 0.05$ versus WT DJ-1 (P/M, pyruvate/malate; Rot, rotenone).

Viral expression of Pink1 was previously described (32). As shown in Figure 7A and C and quantified in Figure 7B and D, overexpression of either Pink1 or Parkin in DJ-1^{-/-} primary cortical neurons promoted an increase in the percentage of mitochondria that were greater than 3 μ m in length ($5.151 \pm 0.826\%$ in KO-GFP versus $44.08 \pm 1.646\%$ in KO-Pink1; Fig. 7B and $1.686 \pm 0.133\%$ in KO-GFP versus $30.126 \pm 8.068\%$ in KO-Parkin; Fig. 7D) and decreased the percentage of fragmented mitochondrial (i.e. $<0.5 \mu$ m; $42.00 \pm 2.562\%$; Fig. 7B in KO-GFP versus $0.948 \pm .271\%$ in KO-Pink1 and $34.108 \pm 5.50\%$ in KO-GFP versus $4.888 \pm 2.924\%$ in KO-Parkin; Fig. 7D) respectively, suggesting that both Pink1 and Parkin can rescue the fragmentation phenotype observed with the loss of DJ-1.

To further confirm these findings, we also quantified the percentage of cells that contained fragmented mitochondria a dopaminergic cell line (SH-5Y5Y) in which DJ-1 was transiently knocked down and subsequently overexpressed with Parkin or Pink1. Confirmation of DJ-1, Pink1 and Parkin overexpression is shown in Supplementary Material, Fig. S4A, S4B and S4C, respectively. As seen in DJ-1^{-/-} primary cortical neurons, transient knockdown of DJ-1 produced a significant increase in cells exhibiting fragmented mitochondria and this phenotype could be prevented with overexpression of DJ-1, Pink1 or Parkin (Fig. 7E and F).

DJ-1 deficiency results in enhanced autophagic flux

As mentioned above, Pink1 and Parkin have both been implicated in the regulation of autophagy in response to mitochondrial damage (9–11,30,31). Our present data show that the loss of DJ-1 leads to increased mitochondrial ROS production and fragmentation. Since both of these parameters are linked with autophagy, we evaluated whether a downstream autophagic response might also be altered with DJ-1 deficiency. To this end, we employed conventional autophagy assays including the evaluation of steady-state microtubule-associated protein light chain 3-II (LC3-II) and the LC3-associated protein p62, under basal conditions as well as GFP-LC3 puncta formation (33). As shown in Figure 8A, the markers

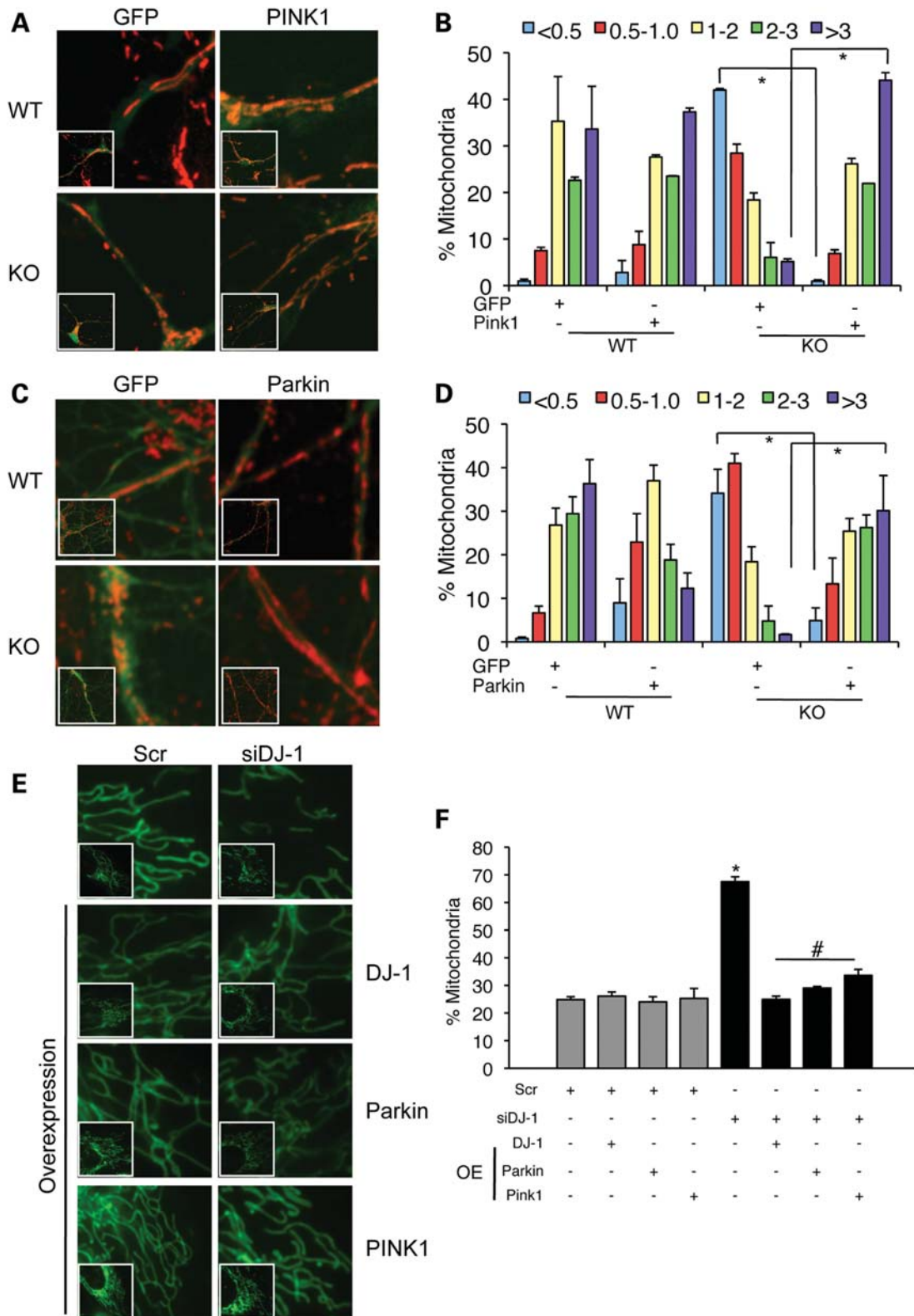


Figure 7. Rescue of mitochondrial morphology with Pink1 and Parkin in a DJ-1-deficient background. (A) Confocal images from WT and KO DJ-1 primary cortical neurons infected with GFP or GFP-PINK1 adenoviruses as described in Materials and Methods. Neurons were harvested and fixed 48 h post-infection and immunostained with antibodies to Tom20 (red) to visualize mitochondria. Inset: lower magnification images. (B) Quantification of mitochondrial lengths as described previously [Jahani-Asl *et al.* (20); $n = 3$ independent experiments with a minimum of 500 mitochondria/experiment that were counted per condition]. Scale bar = 5 μm . * $P < 0.05$ versus respective controls. (C) Confocal images from WT and KO DJ-1 primary cortical neurons infected with eGFP or eGFP-Parkin adeno-associated viruses as described in Materials and Methods. Neurons were harvested and fixed 4 days post-infection and immunostained with

of autophagy p62 and LC3-II levels in DJ-1^{-/-} mouse embryonic fibroblasts (MEFs) are decreased compared with DJ-1^{+/+} controls, indicating that the loss of DJ-1 results in a reduction in autophagosomes since levels of LC3-II correlate with autophagosome number (34,35). This reduction can either be attributed to the downregulation of autophagosome formation or enhanced autophagic degradation (34). The use of bafilomycin A1, a late inhibitor of autophagy (34), restores steady-state p62 as well as LC3-II protein levels suggesting that the loss of DJ-1 enhances autophagic degradation, in other words autophagic activity is overactive. Next, we made use of the H1299 cell line in which GFP-LC3 is stably expressed and transiently reduced DJ-1 protein levels via siRNA to confirm the DJ-1-dependent perturbations in the autophagic pathway. As shown in Figure 8B, at 48 h post-transfection, the level of DJ-1 was significantly reduced upon transfection of a siRNA specifically targeted to DJ-1. The effect of DJ-1 knockdown was accompanied by a significant decrease in p62 levels, and an increased accumulation of cleaved GFP demonstrating that autophagic activity is enhanced by transient knockdown of DJ-1 (Fig. 8B). This was further observed with immunofluorescence where GFP puncta formation was increased by 1.5-fold ($P < 0.05$) with transient knockdown of DJ-1 (Fig. 8C and D). Given the recent involvement of Parkin and PINK1 in the regulation of mitochondria specific autophagy (mitophagy), we also assessed the steady-state levels of mitochondrial markers to determine whether DJ-1 may also play a role. As shown in Figure 8E, loss of DJ-1 does not induce significantly altering the expression of cytochrome *c* oxidase (COX) subunits of complex I or complex V. Furthermore, expression of the outer mitochondrial membrane marker Tom20 was also unchanged. This suggests that mitophagy, at least at a gross level, is not affected by the loss of DJ-1. This theory is supported by initial observations that Parkin is not significantly recruited to mitochondria in DJ-1 KO cells under basal conditions (Joselin *et al.*, unpublished data).

Mitochondrial morphology and autophagy are also perturbed in human DJ-1-linked Parkinson's disease

Finally, to provide evidence that the DJ-1-dependent perturbations in mitochondrial homeostasis also extend to a human model of DJ-1-linked PD, we obtained human lymphoblasts isolated from control and PD patients. The PD lymphoblasts were obtained from an Italian and Dutch family, respectively (12). The previously described L166P pathogenic mutation found in the Italian family consists of a leucine to proline substitution at amino acid 166, while the Deletion (Del) mutation, found in a Dutch family, results from a complete loss of exons 1–5 (12). As shown in Figure 9A, similar to the pattern of

mitochondrial morphology observed in DJ-1^{+/+} and DJ-1^{-/-} murine tissues, electron microscopic analysis of lymphoblasts isolated from human PD patients (L166P, DEL) contained a greater percentage of fragmented mitochondria compared with control lymphoblasts (i.e. $<0.5 \mu\text{m}$; $41.578 \pm 2.41\%$ and $48.316 \pm 6.02\%$ in PD versus $12.62 \pm 3.03\%$ and $9.755 \pm 2.23\%$ in controls) and a smaller percentage of mitochondria that were longer than $>1.0 \mu\text{m}$ ($23.019 \pm 0.84\%$ and $23.997 \pm 0.94\%$ in PD versus $41.578 \pm 2.41\%$ and $48.316 \pm 6.02\%$ in controls; Fig. 9B). We also evaluated whether autophagy was similarly affected in human DJ-1-linked PD and observed that p62 was decreased in both PD patient cell lines when compared with CTRL lymphoblasts (Fig. 9C). These data confirm that the mitochondrial morphology as well as changes in autophagic markers observed in DJ-1^{+/+} and DJ-1^{-/-} are also present in human DJ-1-linked PD.

DISCUSSION

Mitochondrial dysfunction appears to contribute to the progression of sporadic PD and it has been postulated that excess ROS produced as the result of mitochondrial dysfunction may be an important reason for which neurons exhibit increased sensitivity to oxidative stress-induced neuronal cell death (36,37). Emerging evidence points to underlying defects in mitochondrial morphology and dynamics as a potential mechanism to explain this increased sensitivity (38). In PD, this relationship is significant since several PD-linked genes (DJ-1, Parkin, Pink1) have been found to reside or translocate to the mitochondrial compartments (7,8,29,39–42), participate in mitochondrial remodeling (7,9–11) and actively regulate mitochondrial quality control (18,19,41–43). Of the three PD-linked genes that have been associated with mitochondria, the least is known regarding the role of DJ-1.

Mitochondrial morphology, dynamics and ROS production are altered by the loss of DJ-1

We first began our investigation by characterizing the impact of DJ-1 deficiency on mitochondrial morphology and function under steady-state conditions in a variety of experimental systems. We demonstrated both *in vitro* and *in vivo* neuronal and non-neuronal cells, as well as in brain tissue that mitochondria are significantly more fragmented with the loss of DJ-1. Importantly, we also extended these findings to human DJ-1-linked PD to convincingly implicate that an aberrant DJ-1-dependent mitochondrial phenotype in a more disease relevant model. We also demonstrated that the mitochondrial phenotype produced by the loss of DJ-1 contributes to the

antibodies to Tom20 (red) to visualize mitochondria. Inset: lower magnification images. (D) Quantification of mitochondrial lengths as described previously [Jahani-Asl *et al.* (20); $n = 3$ independent experiments with a minimum of 500 mitochondria/experiment that were counted per condition]. Scale bar = $5 \mu\text{m}$. * $P < 0.05$ versus respective controls. (E) Confocal images from SH-5Y5Y cells in which DJ-1 has been knocked down via siRNA, and transfected with DJ-1, Parkin or PINK1 as described in the Materials and Methods. Inset: lower magnification images. (F) Quantification of at least 300 cells/condition was performed as described in the Materials and Methods. Data are representative of at least three independent experiments where each condition was done in triplicate. OE, overexpression. Scale bar = $5 \mu\text{m}$. * $P < 0.05$ versus siDJ-1- and # $P < 0.05$ versus siDJ-1.

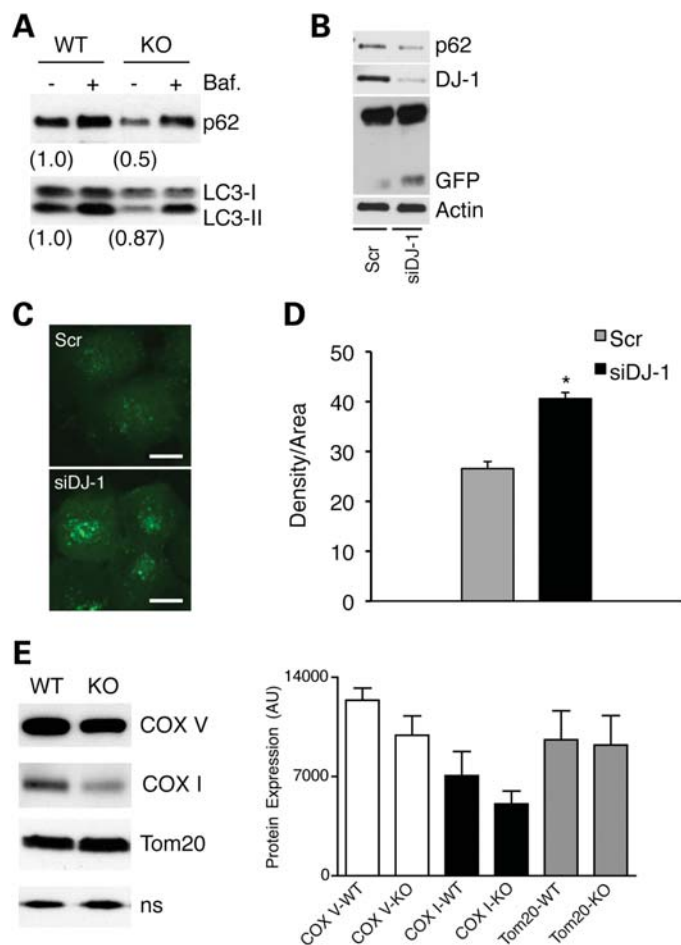


Figure 8. Cells deficient for DJ-1 undergo enhanced autophagic activity. (A) Proteins were extracted from WT and KO DJ-1 MEFs treated with (+) or without (–) Bafilomycin (10 μ g/ml, 3 h) and subjected to western blotting for p62 and LC3-I and LC3-II levels. Numbers below representative images refer to fold changes versus WT (–Baf) after correction for actin. Data are representative of three independent experiments. (B) Total cell lysates from H1299 cells stably expressing GFP-LC3 and transfected with either scrambled (Scr) or a siRNA against DJ-1 (siDJ-1) were analyzed by western blotting for DJ-1, p62 and free GFP. Data are representative of at least three independent experiments. (C) Confocal images of H1299 cells stably expressing GFP-LC3 cells transfected with either scrambled (Scr) or a siRNA against DJ-1 (siDJ-1). (D) The density of GFP puncta in Scr versus siDJ-1 conditions was analyzed in at least 150 cells/condition. Data are representative of three independent experiments * P < 0.05, siDJ-1 versus Scr. control. Scale bar = 2 μ m. (E) Total cell lysates from DJ-1 WT and KO MEFs were subjected to western blotting for COX V, COX I and Tom20 levels (n.s., non-specific band was used as a loading control). Data are representative of five to seven independent experiments.

oxidative stress-induced sensitivity to cell death since reversal of the mitochondrial phenotype by overexpression of DN-Drp1 to rescue mitochondrial fragmentation abrogated neuronal cell death induced by MPP⁺. Is the fragmented phenotype a result of increased fission or decreased fusion? Since mitochondrial fusion rates and the steady state levels of the mitochondrial fusion protein MFN1 are decreased in DJ-1 deficient cells, we would be tempted to speculate that mitochondrial fusion is decreased. However, our results also do not rule out that an increase in mitochon-

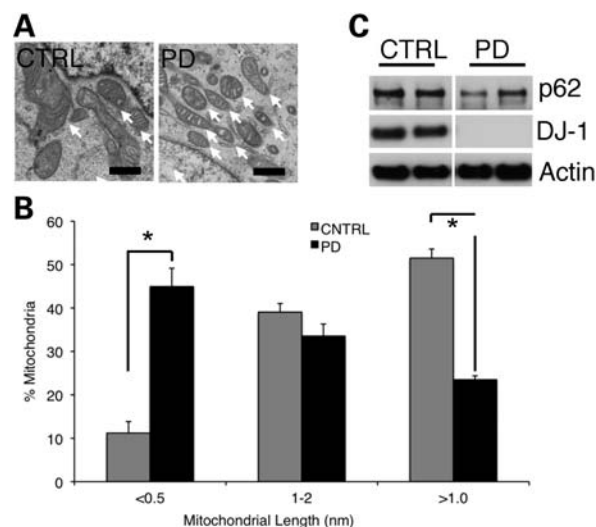


Figure 9. Mitochondrial morphology and autophagy are also perturbed in human DJ-1-linked PD. (A) EM images of mitochondria from human lymphoblasts isolated from healthy control (C48 and GEPA) and PD (L166P and Del) patients. (B) Quantification of mitochondrial diameters in human lymphoblasts (n = 4; at least 200 mitochondria/experiment were counted). Scale bar = 500 nm. * P < 0.05, PD versus control. (C) Total protein was extracted from human control (CTRL), and PD (L166P and Del) lymphoblasts were subjected to western blotting for DJ-1, p62 and actin. Data are quantified as the relative changes in steady state protein levels corrected for loading using actin. Five independent experiments are represented. * P < 0.05, PD versus control. White arrows in (A) depict mitochondria.

drial fission is also a possibility. Indeed, during the preparation of this manuscript, Krebiehl *et al.* (44) demonstrated that altered mitochondrial morphology induced by the loss of DJ-1 could be attributed to changes in mitochondrial fission. The implications of decreased or increased mitochondrial fusion or fission that occurs with DJ-1 deficiency could readily explain the increased sensitivity of these cells to oxidative stress (14), as it is known that fragmented mitochondria precedes apoptosis, or alternatively renders mitochondria more susceptible to death-inducing stimuli (20,45–48).

ROS is important in establishing the DJ-1-dependent phenotype

Based on the known impact of excess ROS on mitochondrial morphology (23), we suspected that the increased ROS produced by mitochondria from DJ-1^{–/–} animals could be responsible for the fragmented phenotype. Indeed, we confirmed this hypothesis by first demonstrating that scavenging ROS with the use of NAC or WT DJ-1 (itself a suspected free radical scavenger), but not an oxidant mutant of DJ-1 (C106A), was able to rescue the fragmented phenotype observed in DJ-1 deficient primary cortical neurons. Our data also show that while the excess H₂O₂ produced within mitochondria by the loss of DJ-1 is sufficient to alter mitochondrial morphology, they are not produced in sufficient concentrations to cause overt changes in mitochondrial oxygen consumption and citrate synthase activity, at least in the

brain. In all likelihood, these factors contribute to the lack of any gross neuronal abnormalities including dopaminergic neuron numbers in the substantia nigra, fiber densities and dopamine levels in the striatum and the absence of any behavioral deficits in untreated DJ-1 deficient mice (14). It is therefore more likely that DJ-1 deficiency compromises the sub-cellular milieu rendering them more vulnerable to additional stress. Indeed, the observation that DJ-1 deficiency does not seem to grossly affect mitophagy leading to the accumulation, instead of the removal of fragmented mitochondria further adds to this possibility. This theory is also supported by initial observations that Parkin is not significantly recruited to mitochondria in DJ-1 KO cells under basal conditions (Joselin *et al.*, unpublished data).

Indeed, as we have previously demonstrated, DJ-1 deficient animals/cells are hypersensitive to MPTP or hydrogen peroxide treatment and this hypersensitization results in the previously described dopaminergic cell death and behavioral deficits, effectively recapitulating some pathological and clinical features of human PD (14).

Pink1 and Parkin can rescue DJ-1 deficient mitochondrial fragmentation

We also assessed the relationship of DJ-1 with Parkin and Pink1, two recessively linked PD genes, as they have all been implicated in regulating aspects of mitochondrial morphology and/or dynamics. Previous studies using the *Drosophila melanogaster* model have shown that the loss of Pink1 and Parkin independently compromise mitochondrial integrity (9,11,28,49). Since double mutants produce an identical phenotype to each mutant alone, and overexpression of Parkin rescues Pink1 deficits but not *vice versa*, it was postulated that they function in the same pathway with Pink1 positioned upstream of Parkin (9,11,28,49). More recently, it has been shown that Pink1 and Parkin actively participate in mitochondrial quality control (18,19,41–43). Given that DJ-1 deficiency induces oxidative stress and mitochondrial defects, we hypothesized that overexpression of these mitochondrial quality control factors would rescue the DJ-1 deficient mitochondrial phenotype. We confirmed this hypothesis using two different models. First, Pink1 and Parkin were overexpressed in DJ-1 deficient primary cortical neurons. In this model, overexpression of either Pink1 or Parkin rescued the fragmented mitochondrial phenotype in DJ-1 deficient cells. Second, we used a dopaminergic cell line to overexpress Pink1 and Parkin in cells where DJ-1 levels were reduced down by siRNA. Similar to our findings in primary cortical neurons, the DJ-1-induced fragmentation phenotype produced by knockdown of DJ-1 was reversed with overexpression of either Pink1 or Parkin. It is important to stress that the exact mechanistic link between DJ-1 and Pink/Parkin is not clear. However, we would propose that DJ-1 somehow modulates the actions or activity of Pink1 and/or Parkin, possibly via its effect on the ROS environment. Given the effects of Parkin and PINK1 deficiency on antioxidant capacity and ROS production (50–52), it is also tempting to speculate that overexpression of either gene could potentially ameliorate that ROS milieu of the DJ-1 deficient cells, thereby reversing

the fragmented phenotype. Alternatively, a more direct regulation is also possible.

DJ-1 deficiency increases autophagic activity

Increasing evidence has implicated several PD-linked genes including Pink and Parkin in the process of autophagy (18,19,41–43). Two recently published studies have now implicated DJ-1 (19,44). We also pursued this phenomenon in the present manuscript and suggest that the loss of DJ-1 promotes enhanced autophagy resulting in increased turnover. According to Mizushima and Yoshimori (34) and Rubinsztein *et al.* (35), a loss in the levels of the autophagy markers LC3-II and p62 at a given time is either attributed to a downregulation of autophagosome formation or enhanced degradation. If the level of LC3-II or p62 rises following incubation with autophagy inhibitors such as Bafilomycin A1, as was seen in the present study, it is considered that during the course of the experimental time frame that the number of molecules degraded exceeds the number being produced. We further assessed the effect of transient DJ-1 knockdown on autophagic activity and found that within 48 h of DJ-1 knockdown, the autophagy was increased, as measured by the decrease in p62 levels and the increase in LC3 puncta formation. It has previously been shown that following acute starvation, autophagy is increased and that prolonged starvation leads to excessive activity and turnover (35). Acute starvation led to decreased p62 levels and LC3 puncta formation, whereas a complete loss of LC3-II levels was observed during prolonged starvation. By analogy, one could interpret that acute DJ-1 knockdown results increase autophagic activity, while germline deletion is associated with excessive autophagic activity resulting in increased turnover. In either condition, autophagic activity is enhanced with DJ-1 deficiency. Future studies will more carefully evaluate the nature of this phenomenon. Additionally, whether DJ-1 more directly regulates the autophagic response or merely influences the ROS environment leading to increased flux is unknown and warrants further study. The evidence suggesting that ROS triggers autophagy would be in keeping with the latter suggestion (53–55). Furthermore, the idea that DJ-1 participates in the Pink1/Parkin pathway temptingly suggests the possibility that DJ-1 could modulate Pink1/Parkin activity and thereby regulate autophagic activity. Alternatively, DJ-1 may more directly regulate additional upstream activators of autophagy, including mTOR and AMPK, which has been suggested previously (19). More careful analyses will be required to validate these possibilities.

In conclusion, this study demonstrates that DJ-1 plays an active role in the remodeling of mitochondria and regulation of autophagy. Cells lacking DJ-1 display a fragmented mitochondrial morphology that can be rescued with ROS scavengers, wild-type DJ-1, Parkin and Pink1. This DJ-1-dependent mitochondrial morphology contributes to oxidative stress-induced sensitivity to cell death since reversal of this mitochondrial phenotype abrogates neuronal cell death. Finally, we also show that DJ deficiency leads to altered autophagy in DJ-1-deficient murine and human cells. We propose that under conditions of oxidative stress, these derangements may account for the reported increased sensitivity to cell death of DJ-1 deficient neurons.

MATERIALS AND METHODS

Antibodies

The following antibodies were used in this study: mouse anti-Drp-1 (BD Transduction), chicken anti-MFN1 (Novus Biological), rabbit anti-MFN2 (Santa Cruz), mouse anti-COX V (Mitosciences), mouse anti-COX I (Mitosciences), rabbit anti-Tom20 (Santa Cruz), rabbit anti-LC3 (Novus Biologicals), guinea pig anti-p62 (ARP), mouse anti-p62 (Santa Cruz), mouse anti DJ-1 (Stressgen), mouse anti-parkin mouse PRK8 (Santa Cruz), anti-PINK1 polyclonal antibody (Novus Biologicals), anti-DJ1 polyclonal antibody (Abcam), mouse anti- β -cactin (Sigma), horseradish peroxidase-conjugated secondary antibodies (Bio-Rad).

Cell lines, transfections, viral infections and plasmids

MEFs and primary cortical neurons were derived from E14.5–15.5 transgenic DJ-1 animals as previously described (14). Immortalized human lymphoblasts obtained from DJ-1-linked PD (Del or L166P) or healthy controls were cultured as described previously (56). H1299 cell line stably expressing GFP-LC3 cultured as previously described (57). SH-5Y5Y cells were cultivated as previously described (58). For RNA interference, SH-5Y5Y or H1299 cells were reverse-transfected with Stealth siRNA (Invitrogen) using Lipofectamine RNAiMAX (Invitrogen) or siRNA (Santa Cruz) using siLentFect (Bio-Rad), respectively, according to the manufacturer's instructions. *DNA Constructs (SH-5Y5Y cells)*: Human wild-type (wt) parkin and human wild-type PINK1 were described earlier (49,59). Human wild-type DJ was amplified from a human brain cDNA library and inserted into the pcDNA3.1 vector (Invitrogen). *Viral plasmids and infections (primary cortical neurons)*: for rescue studies, cortical neurons were harvested from DJ-1^{+/+} or DJ-1^{-/-} littermate embryos (produced by a heterozygote cross) at E15.5 and plated at a density of 150 000 cells per well (24-well dish) on glass cover slips coated with 1XPoly-D-Lysine. Viral particles expressing GFP, DJ-1, DJ-1 C106A, Pink1 or Parkin were administered at a multiplicity of infection (MOI) of 30 at the time of plating. Cortical neurons infected with DJ-1, DJ-1C106A and Pink1 were harvested 48 h following infection. Cortical neurons infected with Parkin were harvested 4 days post-infection and plating. For cell survival studies, cortical neurons harvested as described above were infected with either control (EGFP) adenovirus or dominant-negative Drp-1 (ECFP-C1 DLVP K38E) adenoviruses at MOI of 40 and then immediately seeded into 24-well plates at an approximate density of 350 000 neurons/well. Neurons were cultured for 3 days and then treated with 10 μ M MPP⁺ for 24 h.

Cell survival

Neuronal survival was evaluated by assessing nuclear integrity of GFP/CFP-positive neurons as done previously (14).

Citrate synthase activity

Maximal activity of citrate synthase (EC 4.1.3.7) was measured at 25°C in previously frozen homogenate and mitochondria from brain and skeletal as previously described (60).

Confocal microscopy/immunofluorescence/mitochondrial fusion rates

Confocal images were acquired with a 63 $\times$ objective (1.4) by an inverted Laser Scanning Microscope (LSM510 META, Zeiss). Mitochondrial fusion rates were calculated as previously described (21).

Generation and genotyping of DJ-1 mice

The generation and genotype of the DJ-1 deficient mice has previously been described in detail (61).

H₂O₂ generation

Mitochondrial H₂O₂ production rate was determined in freshly isolated mitochondria from skeletal muscle and brain using the *p*-hydroxyphenylacetate (PHPA) fluorometric assay (62). Mitochondria (0.1 mg/ml) were incubated in standard incubation medium (IM: 120 mM KCl, 1 mM EGTA, 5 mM KH₂PO₄, 2 mM MgCl₂ and 3 mM HEPES; pH 7.4) supplemented with 0.3% defatted BSA. H₂O₂ production was monitored for up to 25 min using a temperature-controlled fluorimeter (BioTek, FLx800) at 37°C. Fluorescence readings were converted to H₂O₂ production rates by use of a standard curve.

Immunofluorescence (primary cortical neurons and MEFs)

Cortical neurons or MEFs were fixed with 4% PFA diluted in cell culture medium for 15 min at 37°C. Cells were then washed 3 $\times$ with 1XPBS. Immediately following this, cells were permeabilized and blocked with 10% normal goat serum-0.1% Triton X/PBS for 1 h at room temperature. Cells were then stained with Tom-20 (1:100, a kind gift from Dr Gordon Shore or from Santa Cruz) or *cytochrome c* (1:100, BD Biosciences, in 5% normal goat serum overnight at 4°C) for the visualization of mitochondria. The following day, cells were washed 3 $\times$ with 5% normal goat serum/PBS and then incubated for 1 h with the appropriate Alexa conjugated fluorophores in 5% normal goat serum/PBS. Cells were then washed 3 $\times$ with 1XPBS, rinsed in sterile H₂O and mounted onto microscope slides using Gel Mount (Sigma).

Fluorescent staining of mitochondria and western blot analysis (SH-5Y5Y cells)

SH-5Y5Y cells were grown on 15 mm glass cover slips. Cells were fluorescently labeled with 0.1 μ M DiOC₆ (3) in cell culture medium for 15 min. After washing the cover slips with medium, living cells were analyzed for mitochondrial morphology by fluorescence microscopy using a Leica DMRB microscope (Leica, Wetzlar, Germany). Cells were categorized in two classes according to their mitochondrial morphology: tubular or fragmented. Cells displaying an intact network of tubular mitochondria were classified as tubular. When this network was disrupted and mitochondria appeared predominantly spherical or rod-like, they were classified as fragmented. The mitochondrial morphology of at least 300 cells per plate was determined in a blinded manner, i.e. the researcher was blind to the transfection status.

Quantifications were based on triplicates of at least three independent experiments. Proteins were analyzed by SDS–PAGE and western blotting using polyvinylidene difluoride membranes (Millipore, Schwalbach, Germany). The membranes were blocked with 5% non-fat dry milk in TBS containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature and then incubated with the primary antibody in blocking solution for 16 h at 4°C. After extensive washing with TBS-T, the membranes were incubated with HRP-conjugated secondary antibody for 60 min at room temperature. Following washing with TBS-T, the antigen was detected with the enhanced chemiluminescence (ECL) detection system or ECL plus detection system (Amersham Biosciences, Freiburg, Germany).

Immunoblotting

Cell lysis was carried out identically for both MEFs and neurons. Cells were washed twice with PBS, scraped in lysis buffer containing 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 0.4% Triton X-100, 1 mM DTT and 1× protease inhibitor cocktail (Roche). Samples were kept on ice for 20 min and then spun with maximal speed at 20 000g at 4°C for 5 min. Protein quantification was carried out using both Bradford (Bio-Rad) and BCA (Pierce) methods. Fifteen micrograms of each lysate was electrophoresed on 12% SDS–polyacrylamide gels, or 4–20% gradient gels (Invitrogen) and transferred to polyvinylidene fluoride (PVDF) or nitrocellulose membranes (Millipore). For tissue lysates, 15 µg of each tissue lysate was electrophoresed on 12% SDS–PAGE gels and transferred to polyvinylidene fluoride (PVDF) or nitrocellulose membranes (Millipore).

Isolation of mitochondria

DJ-1^{+/+} or DJ-1^{-/-} mice (4–6 months old) were euthanized by decapitation for isolation of skeletal muscle and brain mitochondria. Isolation of skeletal muscle mitochondria was performed using a modified method of Chappell and Perry (63), as previously described in detail (64). Brain mitochondria were isolated as described (50).

Lentivirus production and transduction

Lentiviral vectors were generated by transient transfection in 293T cells using PEI. The constructs for manufacturing the lentiviruses were obtained from Addgene.org. Protocols used to manufacture and purify lentiviruses were done according to Tronolab's protocols (www.tronolab.com).

Oxygen consumption

Oxygen consumption was measured in isolated brain mitochondria (0.3 mg/ml) at 37°C using a Clark-type oxygen electrode (Hansatech, Norfolk, UK), incubated in standard incubation medium (IM: 120 mM KCl, 1 mM EGTA, 5 mM KH₂PO₄, 2 mM MgCl₂ and 3 mM HEPES; pH 7.4) containing 0.3% defatted BSA and assumed to contain 406 nmol O/ml at 37°C (65). State 3 (maximum phosphorylating) respiration was determined using 5 mM glutamate/5 mM malate as substrate, and 500 µM ADP. State 4 (non-phosphorylating or

maximal leak-dependent respiration) was determined following addition of oligomycin (8 µg/ml). All measurements were performed in duplicate.

Statistical analyses

Unless otherwise described, data analysis was carried out using independent two-tailed *t*-tests. Significance was marked by * when *P* < 0.05. All data are presented as means ± SEM.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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