The Role of Parkinson's Disease Gene PTEN-Induced Putative Kinase 1, PINK1 in Ischemia Induced Neuronal Injury

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Abstract

Stroke results from disturbance in blood flow to an area of the brain, leading to neuronal dysfunction and loss. Mitochondrial dysfunction and oxidative stress are critical factors in neuropathology of stroke. They have also been implicated in Parkinson's disease (PD). Select cases of PD are caused by homozygous mutations in the PINK1 gene. Critically, this gene works with another PD gene, Parkin, to regulate mitochondrial quality control (MQC) mechanisms. Additionally, initial studies of the PINK1 protein have suggested that it plays a critical role in cellular pro-survival responses to oxidative stress though the mechanism by which it does so is unclear. In this dissertation, I explored the potential mechanisms through which PINK1 confers neuroprotection, particularly in the case of ischemic insult. I found that PINK1 deficiency sensitizes neurons to glutamate-induced excitotoxicity. I also found that the PINK1 kinase domain, but not the mitochondrial targeting motif, is essential for its protective effect. Additionally, PINK1 or Parkin deficiency significantly increases the infarct volume after middle cerebral artery occlusion, *in vivo*. Importantly, expression of Parkin reduces the sensitivity of neurons to cytotoxicity induced by PINK1 deficiency indicating that Parkin functionally interacts with PINK1 either through the same or on parallel survival pathways. Moreover, I investigated if PINK1 and Parkin confer neuroprotection against ischemia through PINK1/Parkin MQC pathways. However, I did not find any evidence indicating Parkin mitochondrial translocation following stroke insult suggesting that PINK1/Parkin MQC pathways are not involved in the protective functions of PINK1/Parkin. Interestingly, I found that PINK1 or Parkin deficiency decreases the level of phosphorylation of pro-survival protein AKT (pAKT) whereas expression of these genes enhances pAKT following glutamate treatment.

My data also indicate that the mTORC2/AKT pathway partially mediates the neuroprotective effect of PINK1. Taken together, my data indicate that both PINK1 and Parkin play a critical neuroprotective role against ischemia and Ca^{2+} dysregulation in a fashion independent of mitochondrial control but dependent on AKT function.

Table of Contents

Table of Figures

List of Abbreviations:

µl	micro litter
µM	micro Mole
AAV	Adeno Associated Virus
AFG3L2	AFG3-Like AAA ATPase 2
AIF	Apoptosis Inducing Factor
AKT	Protein Kinase B (PKB), Ak strain with Thymoma
ALS	Amyotrophic Lateral Sclerosis
AMPA	amino-3-hydroxy-5-methyl-4-isoxazolepropionate
ANOVA	Analysis of Variance
Apafl	Apoptotic protease activating factor-1
Ara*C*	Cytosine-1-β-D-arabinofuranoside
ATP	adenosine-5'-triphosphate
AV	Adenovirus
Bax	Bcl-2-associated X protein
Bid	BH3 interacting-domain death agonist
BSA	Bovine Serum Albumine
CA1	Cornu Ammonis 1
CCCP	Carbonyl Cyanide m-chlorophenylhydrazone
CGN	Cerebellar Granule Neurons
CNS	Central Nervous System
COX	Cyclooxygenase
DNA	Deoxyribonucleic acid

Drp1	Dynamin-related protein1
e.g.	For instance
GFP	Green Fluorescent Protein
GI	GastroIntestinal
GLU	Glutamic acid
GSH	Glutathione
GTPase	Guanosine triphosphatase
hr(s)	hour(s)
Hsp75	Heat shock protein 75
HtrA2	High temperature requirement protein A2
IBR	In-between ring
IL	Interleukin
kDa	kilodalton
Ko	Gene knock Out
LRRK2	Leucine Rich Repeat Kinase 2
Marf	Mitochondria assembly regulatory factor
MCAO	Middle Cerebral Artery Occlusion
Mfn	guanosine triphosphatase mitofusin
min	minute
Miro	Mitochondrial RHO GTPase
mM	milli Mole
MPP	Mitochondrial Processing Peptidase
MPP^+	1-methyl-4-phenylpyridinium ion

MPTP	1 -methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mTOR	mammalian Target Of Rapamycin
mTORC2	mammalian Target Of Rapamycin complex 2
n	sample size
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NMDA	N-methyl D-aspartic acid
NO	Nitric oxide
NOS	Nitric Oxide Synthase
ONOO-	Peroxynitrite
PARL	Presenilins-associated rhomboid-like
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate Buffered Saline
PD	Parkinson's Disease
PDK1	3-Phosphoinositide Dependent protein Kinase-1
PFA	ParaFormAldehyde
PH (domain)	Pleckstrin Homology
PI3K	Phosphatidyl Inositol 3-Kinase
PINK1	PTEN Induced Kinase 1
PIP2	Phosphatidyl Inositol 4,5-bisphosphate
PIP3	Phosphatidyl Inositol (3,4,5)-triphosphate
PRX	Peroxiredoxin
PTEN	Phosphatase and TENsin homolog
Rap	Rapamycin

RNA	Ribonucleic acid
RNAi	RNA interference
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SNc	Substantia Nigra *pars compacta*
SOD	Superoxide Dismutase
TIM	Translocase of the inner membrane
TNF	Tumor Necrosis Factor
TOM	Translocase of the outer membrane
tPA	tissue-Type Plasminogen Activatior
TRAP1	TNF-receptor-associated protein 1
UBL	Uubiquitin like
UCP	UnCoupling Protein
vs	versus
WT	Wild Type

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Lastly, I would like to dedicate this thesis to my father and my mother, a continual source of love and inspiration through my entire life. I feel so fortunate and honored for having you in my life. Love you all!

List of Manuscripts

Farzaneh Safarpour, Mandana Amini, Wassamon Boonying, M.Emdadul Haque, Ali Hage, Jie Shen, David S. Park, The Parkinson's disease genes PINK1/Parkin rescue stroke-induced neuronal damage, ***In Preparation***

Appended Articles

I. Qu D., Hage A., Don-Carolis K., Huang E., Joselin A., **Safarpour F.**, Marcogliese P.C., Huang T., Im D., Callaghan S., Dewar-Darch D., Figeys D., Slack R.S., Shen J., Park D.S., Bag2 mediated regulation of Pink1 is critical for mitochondrial translocation of Parkin and neuronal survival, J Biol Chem. 2015 Nov 4. pii: jbc.M115.677815. [Epub ahead of print]

II. Iyirhiaro G.O., Zhang Y., Estey C., O'Hare M.J., **Safarpour F.**, Parsanejad M., Wang S., Abdel-Messih E., Callaghan S.M., During M.J., Slack R.S., Park D.S. (2014), Regulation of Ischemic Neuronal Death by E2F4-p130 Protein Complexes. J Biol Chem. Jun 27;289(26):18202-18213

III. Haque M.E., Mount M.P., **Safarpour F.**, Abdel-Messih E., Callaghan S., Mazerolle C., Kitada T., Slack R.S., Wallace V., Shen J., Anisman H., Park D.S. (2012) Inactivation of Pink1 in vivo sensitizes dopamine producing neurons to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and can be rescued by autosomal recessive PD genes, Parkin or DJ-1. J Biol Chem. 29;287(27):23162-70

Thesis format

This thesis has been prepared in agreement with the guidelines set forth by the Department of Cellular and Molecular Medicine, Neuroscience program; Faculty of Medicine, University of Ottawa. It has been written as a collection of 4 chapters, starting with a general introduction reviewing the literature surrounding the thesis topic and our current knowledge in the field. The second chapter provides readers with the technical specification related to the present thesis work including the methods and materials applied in this research study. The third chapter contains the results of the thesis project. The final chapter presents the general discussion on the thesis findings as well as other findings in the field.

Chapter one, the general introduction, presents a brief summary of stroke and the processes governing neuronal death following an ischemic attack. It also reviews the current body of knowledge surrounding Parkinson's disease (PD) research. In addition, PINK1, as a Parkinson`s gene, is introduced to the reader and its important pro-survival role is also outlined. This chapter further provides background knowledge and rationale for chapter three describing the experimental work.

Chapter two presents a detailed description on the methods and materials employed in this thesis project.

Chapter three presents the body of the doctoral work. This chapter is devoted to elucidation of the critical role of Parkinson`s disease gene PINK1 in defence against ischemia-induced neuronal damage. This chapter also, provides strong evidence for the contribution of another Parkinson`s disease gene, Parkin in adult models of ischemia. We have also provided

functional evidence for involvement of the AKT pathway as the mediator of pro-survival function of PINK1 and Parkin.

Chapter four summarizes the data contained in chapter three and presents the interpretation of the findings. Furthermore, a discussion on the future directions that need to be undertaken with regards to our findings is presented.

Chapter 1

Introduction

1. Introduction:

1.1 Stroke:

1.1.1. Etiology and pathogenesis of stroke:

Stroke results from disturbance in blood flow to an area of the brain leading to neuronal dysfunction and loss. Based on the cause of the disease, stroke is primarily classified into two broad categories: ischemic and hemorrhagic strokes. Ischemic stroke is caused by an interruption of blood flow due to a thromboembolic event whereas; hemorrhagic stroke is caused by bleeding of an artery to the brain. In both conditions, affected neurons die mainly due to lack of oxygen and glucose. Following a stroke, a number of innate pro-survival and pro-death pathways are activated and the final balance between them determines neuronal fate. The outcome of activation of these paradoxical pathways is determined by the severity, duration and location of the ischemic insult. Accordingly, neurons in the core of an infarct region undergo a necrotic or more rapid excitotoxic type of cell death. However, neurons in the surrounding tissue, known as the stroke penumbra, survive acute ischemic injury for a limited period of time. In fact, if blood flow is restored during the early hours of a stroke, the penumbra has a potential for recovery but, if it is left untreated, the neurons exhibit a programmed or apoptotic type of death (Maulaz et al., 2005). Therefore, penumbra is considered to be the main target for therapeutic interventions in a narrow time window.

1.1.2. Epidemiology of stroke:

Stroke is one of the most common leading causes of death and disability in Canada. Approximately, every 10 minutes a Canadian suffers a stroke. That is estimated 50,000 strokes per year. Almost one third of these strokes cause death within the first year, and a further 30% are disabling. Worldwide, 5.5 million people die from stroke every year (http://www.who.int/cardiovascular_diseases/resources/atlas/en/).

Patients who survive a stroke require expensive care, which is a great burden for the Canadian health-care system. The cost of the direct and indirect health-care for the patients, just in the first six months after they are hit by a stroke, exceeds more than $2.5 billion a year in Canada (Mittmann et al., 2012). (http://www.heartandstroke.on.ca/site/c.pvI3IeNWJwE/b.3581729/k.359A/Statistics.htm#stroke)

1.1.3. Pathophysiology of Stroke:

Glucose is the primary source of energy for the brain. In fact, aerobic metabolism of glucose is the predominant source of energy for our brain (Goyal et al., 2014). This energy is utilized for the maintenance of ionic gradients, synaptic activity and biosynthesis. Therefore, constant and adequate blood supply is necessary to meet the brain's high demand for energy (Deb et al., 2010; Markus, 2004; Moustafa and Baron, 2008). Interruption of blood flow rapidly leads to energy depletion, and consequently ionic imbalance and neuronal depolarization. These initial dysfunctions trigger multiple interplaying pathways including but not limited to exitotoxicity, oxidative stress, mitochondrial dysfunction,

protein aggregation, and neuroinflammation (Martin et al., 1994). Among these pathways, excitotoxicity plays a key role in initiation and progression of ischemia-induced neuronal injury.

Excitotoxicity is defined as excessive activation of neurons by excitatory neurotransmitters such as glutamate. Primarily, excitotoxicity is initiated by neuronal depolarization, which leads to massive release of glutamate from nerve terminals into the synaptic cleft (Dirnagl et al., 1999; Dong et al., 2009). Due to the energy depletion caused by stroke, astrocytes fail to remove the excessive glutamate from the extracellular space. The failure of astrocytic in uptaking of glutamate and the abnormal ionic gradient lead to constant activation of glutamate receptors including the N-Methyl D Aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-propionate (AMPA) and metabotropic glutamate receptors (Brouns and De Deyn, 2009; Durukan and Tatlisumak, 2007; Mergenthaler et al., 2004). Intensive activation of the NMDA receptors leads to massive influx of Ca^{2+} and excitotoxicity. Additionally, activation of the AMPA receptors results in excessive intracellular Na^{+} and Cl^{-} ions, which in turn induces passive influx of water, and therefore brain edema (Dirnagl et al., 1999; Dong et al., 2009).

Excessive intracellular Ca^{2+} potentially activates various enzymes such as endonucleases, proteases, phospholipases, which can seriously damage the cellular components. Additionally, Ca^{2+} activates cyclooxygenases (COXs) and nitric oxide synthase (NOS) (Moro et al., 2005). These two enzymes along with other inflammatory

factors such as cytokines (IL-1, IL-6, TNF-a) and gliosis are part of the destructive inflammatory responses induced by ischemia (Ceulemans et al., 2010).

Theoretically, the crisis initiated by ischemia could be reversed by returning the blood flow to the affected area. However, the paradoxical consequences of reperfusion exacerbate the brain damage by inducing oxidative stress (Saito et al., 2005). Oxidative stress is defined by the cellular damage induced by excessive production of reactive oxygen species (ROS). In fact, ROS are normal by-products of oxygen metabolism. These oxidizing radicals are highly reactive, and could cause severe damage to proteins, lipids and DNA, and consequently lead to neuronal death (Valko et al., 2007). Cells normally have a number of defence mechanisms by which they scavenge intracellular ROS (see below). Oxidative stress happens when intracellular anti-oxidant mechanisms are overwhelmed by the high level of ROS production.

Mitochondria are one of the major sources of ROS production. During oxidative phosphorylation in mitochondria, electron transport chain utilizes the oxygen consumed by cells for electron transfer and ATP synthesis (Moro et al., 2005). It has been shown that electrons can leak from the electron transport chain in inner mitochondrial membrane (Adam-Vizi, 2005). These leaky electrons target O_2, and reduce it to superoxide anion ($\bullet O_2^-$). Superoxide anion is highly reactive. Under normal conditions, mitochondrial superoxide dismutase (Mn-SOD or SOD2) rapidly catalyzes reduction of superoxide anion to hydrogen peroxide (H_2O_2), which is a mild oxidizing agent. H_2O_2 is normally removed by activity of

anti-oxidant enzymes such as glutathione (GSH) and peroxiredoxin (PRX) (Birben et al., 2012; Moro et al., 2005).

The normal balance between antioxidant defense systems and production of ROS is disturbed in many neurological conditions such as ischemia. During the course of ischemia/reperfusion, production of superoxide anion ($\bullet O_2^-$) dramatically increases when oxygen interacts with uncoupled complexes I and III in damaged mitochondrial electron transport chains (Birben et al., 2012; Moro et al., 2005). In the presence of metal ions such as Fe^{2+} or Cu^+, superoxide anion ($O_2^{\cdot-}$) can react with H_2O_2 to break it down to hydroxyl radical (•OH) which is the most reactive ROS (Birben et al., 2012). Additionally, superoxide anion ($O_2^{\cdot-}$) can react with nitric oxide (NO) to produce peroxynitrite (ONOO-) which is a reactive nitrogen species (RNS). These free radicals interact with lipid and protein components of mitochondrial membrane, and as a result interfere with mitochondrial function (Szabo et al., 2007). Nitration of mitochondrial complexes and mitochondrial permeability transition pore components, such as voltage-dependent anion channel, further increases ROS production. Oxidative stress reduces the threshold for opening of mitochondrial permeability transition pore through a vicious cycle, which permits the release of mitochondrial pro-apoptotic factors such as cytochrome *c* and AIF to cytosol. Eventually cytochrome-c-dependent apoptosis is trigged in the cytosol (Szabo et al., 2007).

Together, disturbances in ion homeostasis, intracellular calcium overload, mitochondrial damage and oxidative stress are common features of ischemia. In severe

cases, ischemic neuronal injury ends in edema and necrosis. In mild prolonged ischemic insults, calcium dysregulation causes further damage to the mitochondria, and more production of ROS through a vicious cycle. Eventually, cytochrome c is released from severely damaged mitochondria to the cytosol where it activates the caspase cascade, which triggers apoptotic neuronal death (Dirnagl et al., 1999; Dong et al., 2009) **(Fig.1.1)**.

Figure 1.1. Schematic representation of pathophysiological mechanisms activated by ischemia induced neuronal insult. Energy failure in the ischemic brain leads to neuronal depolarization. Presynaptic neuronal depolarization causes excessive release of glutamate to the synaptic cleft. Activation of post-synaptic glutamate receptors permits massive influx of Ca^{2+}, Na^{+} and Cl^{-} ions into the cytosol while K^{+} is released into the extracellular space. Water shifts to the intracellular space by osmotic gradients induced by Na^{+} and Cl^{-} leading to cell swelling (edema). Intracellular Ca^{2+} overload induces excitotoxicity through over-activation of numerous calcium dependent enzymes such as proteases, lipases, endonucleases, etc. Excitotoxicity leads to mitochondrial damage and generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which further damage lipid bilayer membranes (lipolysis), mitochondria and DNA. Inflammatory reaction in response to RNS gliosis which in turn aggravates the crisis by activation of blood-borne inflammatory responses (Dirnagl et al., 1999).

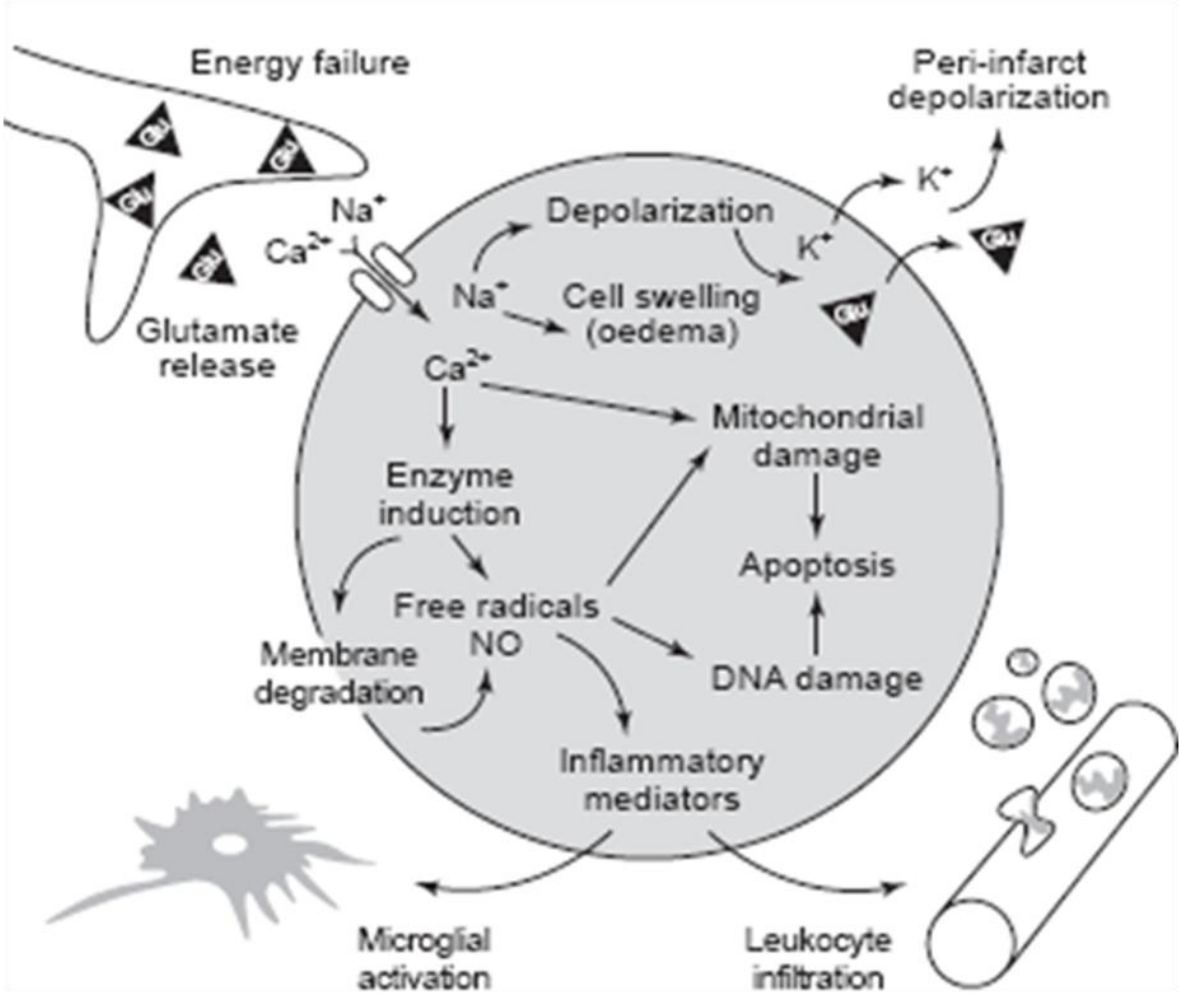

Energy failure
Peri-infarct depolarization
Na+
Ca2+
Depolarization
K+
K+
Glu
Glu
Glu
Glu
Glu
Na+
Cell swelling (oedema)
Glutamate release
Ca2+
Mitochondrial damage
Enzyme induction
Apoptosis
Free radicals NO
Membrane degradation
DNA damage
Inflammatory mediators
Microglial activation
Leukocyte infiltration

Figure 1.1

1.1.4. Characteristics of neuronal death in cerebral ischemia

Characterization of the pathways implicated in ischemic neuronal death is crucial to design effective therapeutic strategies. Neuronal death in ischemia was originally considered to be almost exclusively governed by the process of necrosis. In fact, necrosis is the dominant type of neuronal death in the core of infarct where the blood flow falls under 20% of its normal (Sims and Muyderman, 2010). Necrosis is characterized by cellular changes including organelle swelling, disruption of the plasma membrane and release of intracellular contents. DNA fragmentation is also happening in the late stage of necrosis process (Dong et al., 1997). Although no cellular mechanism appears to be actively regulated during necrosis to induce neuronal death, dysregulation of some pathways such as Poly (ADP-ribose) polymerase (PARP) has been reported to be involved in necrotic death (Hegedus et al., 2008; Szabo et al., 2007).

Unlike necrosis, apoptosis orchestrates a number of cellular pathways to execute cellular death. In focal ischemia, apoptotic neuronal death is more prominent in the penumbra where the blood flow drops down to 20-40% of its normal (Sims and Muyderman, 2010). The complex picture of apoptosis is characterized by DNA fragmentation, apoptotic bodies formation and nuclear condensation (Kroemer et al., 2007; Li et al., 1995). The apoptotic process is activated via two distinct pathways so called the "intrinsic pathway" and the "extrinsic pathway" (Galluzzi et al., 2009). Caspases are major components in both apoptotic death pathways. The caspase family includes highly conserved cysteine proteases expressed in the cell as inactive forms.

Mitochondria play a central role in the regulation of apoptosis via the “intrinsic pathway”. Upon mitochondrial stress, apoptogenic proteins such as cytochrome *c* and Apoptosis Inducing Factor (AIF) are released from mitochondrial inter-membrane space into the cytoplasm to promote apoptosis. In fact, cytochrome *c* has cellular dual function. In association with mitochondrial electron transfer chain, cytochrome *c* serves as an intermediate electron receptor to promote oxidative metabolism. In the cytoplasm and in the presence of ATP, cytochrome *c* binds to the Apoptotic protease activating factor-l (Apafl) to form a complex referred to as the apoptosome. Subsequently, the apoptosome recruits procaspase-9 (caspase-9 precursor) which is cleaved to its active form of capase-9. Upon activation, caspase-9 targets other members of the caspase family such as caspases-3, 6 and 7 (Rodriguez and Lazebnik, 1999). Later on, these executioner caspases cleave many fundamental and structural proteins such as endonucleases, lamin, spectrin and PARP, resulting in plasma and nuclear membrane degradation, DNA fragmentation and nuclear condensation (Hengartner, 2000).

While caspase proteins play an important role in apoptosis, other mitochondrial proteins such as AIF could also promote apoptotic cell death in parallel to the caspase pathways. In fact, AIF is also released to cytosol in response to mitochondrial stressors. Subsequently, AIF is translocated to the nucleus inducing apoptotic nuclear phenotypes such as chromatin condensation and DNA fragmentation (Plesnila et al., 2004; Susin et al., 1999).

As mentioned above, apoptosis could also be activated through the "extrinsic pathway" where specific ligands bind to specialized cell death receptors such as FAS and Tumor Necrosis Factor (TNF) receptors located in plasma membrane. Through activation of cell death receptors, caspase-8 is activated in cytoplasm which in turn activates caspase 3 to execute apoptosis via a mitochondria- independent pathway (Sims and Muyderman, 2010).

In ischemia, apoptosis is induced by multiple factors such as excessive free radicals, DNA damage, death-receptor ligation, p53 induction, growth factor deprivation, TNF production, and cytochrome *c* release from mitochondria (Sims and Muyderman, 2010). The transcription factor p53 is considered the master of cell death through apoptosis (Schmitt et al., 2002). A number of pro-apoptotic pathways have been shown to be induced by p53 in response to oxidative stress. An example of one of these pathways is the involvement of pro-apoptotic members of the Bcl-2 family of proteins, such as BH3 interacting-domain death agonist (Bid) and Bcl-2-associated X protein (Bax) (Culmsee and Mattson, 2005).

In normal healthy neurons, these proteins are sequestrated in the cytosol by anti-apoptotic members of the family such as Bcl-2. Upon oxidative damage, the Bcl-2 pro-apoptotic proteins are translocated to the mitochondria outer membrane. Through interaction with other pro-apoptotic proteins such as Bid, Bim and PUMA, Bax has been shown to be involved in the permeabilization of the mitochondrial outer membrane, and release of apoptogenic proteins (Borner, 2003; Niizuma et al., 2009). The pro-apoptotic

proteins induce apoptosis by pore formation in the mitochondrial membrane leading to release of cytochrome *c* into the cytosol (Adams and Cory, 2001; Kaufmann et al., 2001).

In addition to Bcl-2 family, p53 has been shown to induce a number of other pro-apoptotic proteins such as p53-upregulated modulator of apoptosis (PUMA) (Culmsee and Mattson, 2005). PUMA, which contains two putative p53 binding sites, has been identified as a strong indicator of apoptosis. Similar to Bcl2 family members, PUMA induces apoptosis through a mitochondria-dependent pathway (Nakano and Vousden, 2001). PUMA has been shown to interact with both pro-apoptotic Bax and anti-apoptotic Bcl-2 or Bcl-XL through a BH3 domain to promote apoptosis by cytochrome c release and caspase activation, as previously described (Culmsee and Mattson, 2005).

The above-mentioned pathways are just a few examples of the pathways involved in the pathophysiology of ischemia. These pathways have been briefly discussed to highlight the central role of mitochondrial dysfunction in cellular response to ischemia. Delving into all of the details related to these complex pathways is beyond the scope of this thesis.

1.1.5. Treatment strategies:

Numerous pharmacological compounds and therapeutic strategies have been assessed in clinical trials. Most of them have been focused on re-canalizing occluded vessels by surgical intervention or pharmacological therapies using thrombolytic or anticoagulant compounds. In some of these clinical trials, the efficiency of pharmacological compounds which have anti-oxidant effect or target cytotoxicity by glutamate, AMPA,

GABA, glycine, and ion channel antagonists has been assessed (Vosler et al., 2009). Accordingly, our understanding of the disease and its treatment strategies has been remarkably improved in the last few decades. However, there is no medical or surgical intervention to effectively cure the disease. Most current standard treatments for stroke are involved in alleviation of the symptoms and not the cure of the disease.

Among them, tissue plasminogen activator (tPA) has been the most important break-through in stroke treatment (1995; Kwiatkowski et al., 1999). However, effectiveness of tPA is limited to a very short window of time. Additionally, because of some medical conditions such as subarachnoid hemorrhage or high blood pressure, many patients are not good candidates for this kind of treatment (Tsivgoulis et al., 2015). Lack of efficient treatments urges us to explore more pro-survival and pro-death mechanisms activated in the ischemic brain in order to find new targets for therapeutic interventions.

Stroke shares common features of oxidative stress, mitochondrial dysfunction and excitotoxicity with other neurologic conditions such as Parkinson's disease (Abou-Sleiman et al., 2006b; Koutsilieri and Riederer, 2007; Zuo and Motherwell, 2013). We have previously shown that the Parkinson's disease gene DJ-1 plays a significant role in protecting brain tissue against ischemia (Aleyasin et al., 2007). In the present study, we focus on the role of another Parkinson's disease gene, PINK1, on ischemic neuronal damage. As discussed in following paragraphs, PINK1 **(**PTEN- induced putative kinase 1) has a prominent role in defence against oxidative stress, mitochondrial dysfunction and calcium homeostasis. Generally, these factors have been frequently reported to be

implicated in the pathophysiology of Parkinson's disease (Abou-Sleiman et al., 2006b; Koutsilieri and Riederer, 2007; Zuo and Motherwell, 2013). A brief review on different aspects of the disease would clarify the rationale for this study.

1. 2. Parkinson's disease:

Parkinson's disease (PD) is the most common neurodegenerative movement disorder, affecting approximately one percent of the population age 65 and older. Nearly, 100,000 Canadians live with PD (http://www.statcan.gc.ca/pub/82-003-x/2014011/article/14112-eng.htm). The cardinal signs and symptoms of PD are predominantly motor-related and characterized by resting tremor, slowness of movement (bradykinesia), loss of movement (akinesia), muscular rigidity, gait impairment and postural instability. In many cases, non-motor symptoms including cognitive impairment, mood disorders, olfactory dysfunction (hyposmia) and autonomic disturbances such as constipation, orthostatic hypotension and sweating may precede motor symptoms by many years or even decades (Lees et al., 2009).

1.2.1. Pathology of PD:

The pathology of PD is characterized by progressive dopaminergic neuronal loss in substantia nigra *pars compacta* (SNc) located in the midbrain (Lees et al., 2009). Dopaminergic neuronal loss is typically accompanied by Lewy body inclusions, which are abnormal protein aggregates in cytoplasm. However, neuronal degeneration is not limited to the SNc. In fact, pathologic alterations occur in other regions of brain such as olfactory

bulb, anterior olfactory nucleus, dorsal motor nucleus of vagus and *locus ceruleus* (Braak et al., 2004; Haehner et al., 2011; Larsen and Tandberg, 2001). Neurodegeneration of non-dopaminergic systems lead to non-motor symptoms of PD (Braak et al., 2004; Haehner et al., 2011; Larsen and Tandberg, 2001). For instance, neurodegeneration of the *locus ceruleus* has been highly correlated with sleep disturbance in PD patients (Larsen and Tandberg, 2001; Stocchi et al., 1998). In addition, neuronal loss in olfactory system causes anosmia (loss of the sense of smell) in more than 90% of PD cases years or even decades prior to the onset of PD motor symptoms (Haehner et al., 2011). Moreover, degenerative alterations in dorsal motor nucleus and its peripheral projections frequently induce constipation and other gastrointestinal problems in more that 50% of PD cases (Braak et al., 2006). These observations constitute the primary basis of the Braak hypothesis describing the disease progression in accordance with the temporospatial pattern of Lewy body formation in the brain (Braak et al., 2003a; Braak et al., 2003b).

1.2.2. Etiology and Pathogenesis of PD:

In spite of the enormous progress in our understanding of PD during the last decade, the etiology and pathogenesis of the disease is still not well understood. In fact, the vast majority of PD cases are sporadic with unknown causes, whereas a small number of cases are considered familial, with a family history of the disease.

α-synuclein was the first PD gene identified to be linked to Parkinson's disease in three unrelated families of Greek origin in an autosomal dominant inheritance manner (Polymeropoulos et al., 1997). Through genetic linkage analysis of familial PD, several

other PD genes have been identified as highly associated to the disease. Among them Leucine Rich Repeat Kinase 2 (LRRK2) is linked to autosomal dominant and DJ-1, parkin and PINK1 are linked to autosomal recessive traits (Bonifati et al., 2003; Kitada et al., 1998; Valente et al., 2004; Zimprich et al., 2004). It is noteworthy that research on PD of this small minority of patients has significantly contributed to our knowledge of the disease.

Genetic studies together with epidemiological, clinical and biological data indicate that a complex combination of genetic and environmental factors is involved in initiation and progression of PD (Braak et al., 2004; Tanner, 2003; Tanner and Langston, 1990; Thomas and Beal, 2007). The accidental discovery of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) as a neurotoxin inducing acute Parkinsonism provided strong evidence for the role of environmental factors in the pathogenesis of PD (Langston, 1996). Based on this finding, together with data obtained from other studies, the Braak hypothesis suggests a staging system to describe the pattern of progression of the disease. Accordingly, it is hypothesized that pathologic alterations originally occur in the gastrointestinal (GI) and olfactory systems which are two major routes for ingestion of pathogens such as virus particles or toxins. These pathogenic factors initiate and spread the disease pathology to the CNS through the GI nervous plexus (Auerbach and Meissner plexus) residing in the GI epithelium and the olfactory nerve endings coating the nasal epithelium (Braak et al., 2003a; Braak et al., 2003b). Testing the veracity of this hypothesis is currently the target of intense research in this field.

Nevertheless, apart from aging, which is the main susceptibility factor for the disease, several other risk factors are known to be involved in the pathogenesis of PD. These include exposure to heavy metals such as manganese and iron, head trauma, radiation and contact with pesticides such as Rotenone and Paraquat (Lees et al., 2009). Considering the deleterious properties of these factors, a number of animal models have been created to recapitulate Parkinsonism. These laboratory models have provided much insight into the pathophysiology of PD, although they may not reflect all aspects of the disease.

1.2.3. Pathophysiology of PD:

In spite of the progress in our knowledge of the disease, we still do not know which pathophysiologic pathways are mediating the adverse effects of genetic and environmental factors. Data from post mortem examination of PD patients and studies using animal experimental models have revealed that some pathophysiologic conditions such as oxidative stress, mitochondrial dysfunction, neuroinflammation, protein aggregation and protein turn over are persistent features in parkinsonism (Bender et al., 2006; Brodacki et al., 2008; Chung et al., 2001; Liu et al., 2002; Schapira et al., 1989; Schlossmacher et al., 2002). These data are particularly interesting as suggesting that these recurrent factors may be involved in pathogenesis of PD. Research on familial PD genes strongly supports this concept. The following paragraphs are exclusively devoted to autosomal recessive PD genes, PINK1 and Parkin, as the main focus of this study.

1.2.4. PINK1 and the pathophysiology of PD:

PINK1 was first isolated and characterized in cancer cells as a gene responsive to the tumor suppressor PTEN (Unoki and Nakamura, 2001). PINK1 protein contains 581 amino acids with approximate molecular weight of 62 kDa. It is ubiquitously expressed in body tissues with highest expression in heart, skeletal muscle, and testis. PINK1 is a putative serine/threonine kinase with a mitochondrial targeting motif at its N-terminal (Valente et al., 2004). PINK1 is imported into mitochondria through interaction of its mitochondrial targeting motif with Translocase of the inner/outer membrane (TIM/TOM) complexes of mitochondria (Silvestri et al., 2005). Since the identification of PINK1 as a PD gene, several mutations in PINK1 have been found to be linked to the pathogenesis of the disease. Most of the mutations are localized near or within the serine/threonine kinase domain, and result in a loss-of-function effect. Kinase activity appears to be important for PINK1's pro-survival function, however, previous published data from our lab indicated that mitochondrial localization of PINK1 is not necessary for its neuroprotective effect against MPTP-induced apoptosis (Haque et al., 2008; Petit et al., 2005; Sim et al., 2006).

In order to elucidate the function of PD genes, multiple in-vitro and in-vivo experimental models have been created. Among them, Drosophila melanogaster has provided elegant systems to understand the neurodegenerative process in PD context. For instance, it was noted that PINK1 and Parkin deficient flies display similar abnormal phenotypes, including unusual mitochondrial morphology, impaired mitochondrial function and a moderate dopaminergic neuronal loss (Clark et al., 2006; Park et al., 2006; Wang et

al., 2006). It has been further demonstrated that over-expression of Parkin in PINK1 null flies, but not the reverse, rescues these adverse phenotypes, suggesting that PINK1 is upstream of Parkin in the same pathway (Clark et al., 2006; Park et al., 2006; Wang et al., 2006).

PINK1 and its mitochondrial function have since been the focus of intense research in both Drosophila and mammalian experimental models (Gautier et al., 2008; Kitada et al., 2007; Narendra et al., 2010; Venderova et al., 2009). More relevant to the present work, in a pioneer study, PINK1 has been shown to regulate a mitochondrial $Na+/Ca^{2+}$ exchanger in both human and mouse neuronal cells (Gandhi et al., 2009). In support of this, knock down of PINK1, using RNA interference (RNAi), led to accumulation of calcium in mitochondria resulting in ROS production via NADPH oxidase. The mitochondrial oxidative state has been shown to interfere with its respiratory function by inhibiting the glucose transporter, which leads to premature opening of the mitochondrial permeability transition pore, and increase in vulnerability to death (Gandhi et al., 2009). Follow-up studies have confirmed this regulatory function for PINK1, and emphasized that calcium homeostasis is strongly implicated with mitochondrial dysfunction and oxidative stress as key facets of neurodegeneration(Akundi et al., 2011; Gomez-Sanchez et al., 2014; Heeman et al., 2011; Kostic et al., 2015; Marongiu et al., 2009). Furthermore, electron microscopic analysis of lymphoblasts isolated from human PD patients as well as observations of DJ-1 deficient murine tissues has revealed that mitochondrial morphology is perturbed in the absence of DJ-1 (Irrcher et al., 2010; Thomas et al., 2011). They have also shown that H_2O_2 production is increased in mitochondria isolated from DJ-1 deficient mice. Interestingly, over-

expression of PINK1 or Parkin in DJ-1 deficient cortical neurons could rescue the morphological defect of mitochondria (Irrcher et al., 2010).

To this end, a great deal of research has been devoted to elucidate the role of PINK1 loss of function in calcium dysregulation and mitochondrial dysfunction (Akundi et al., 2011; Gomez-Sanchez et al., 2014; Heeman et al., 2011; Marongiu et al., 2009). However, the molecular mechanisms by which PINK1 regulates mitochondrial function to prevent neuronal degeneration remain poorly understood. In the following section, I have reviewed the literature on PINK1 substrates to describe the biochemical and physiological functions of PINK1 within cells to further address the possible mechanisms underlying neuronal damage induced by PINK1 deficiency.

1.2.5. PINK1's substrates

A number of downstream effectors for PINK1 kinase have been identified. The mitochondrial chaperone TNF-receptor-associated protein 1 (TRAP1), also known as heat shock protein 75 (Hsp75), was reported to be the first specific PINK1 downstream effector (Pridgeon et al., 2007). PINK1 has been shown to bind and co-localize with TRAP1 in the mitochondrial inner membrane and inter-membrane space. PINK1, but not PD-associated PINK1 mutants, has been shown to be protective against oxidative stress-induced cell death through phosphorylation of TRAP1 (Pridgeon et al., 2007). In addition, the kinase activity of PINK1 was demonstrated to be necessary for suppression of mitochondrial cytochrome c release and apoptosis (Pridgeon et al., 2007).

Later, a mitochondrial serine protease, high temperature requirement protein A2 (HtrA2), was identified to be indirectly phosphorylated by PINK1 (Plun-Favreau et al., 2007). Indeed, PINK1 forms a complex with mitochondrial HtrA2 thereby enhancing the phosphorylation of HtrA2 by the stress-activated kinase P38γ. Interestingly, loss of function mutation in HtrA2 is implicated in the pathogenesis of Parkinson's disease (Strauss et al., 2005). Consistent with this observation, HtrA2 phosphorylation is reduced in brains of PD patients carrying mutations in PINK1 suggesting that PINK1 plays an important role in regulating the proteolytic activity of HtrA2 to promote cell survival upon mitochondrial damage (Plun-Favreau et al., 2007).

Around the same time a couple of studies published in *Nature* introduced one of the most attractive PINK1 effectors; Parkin (Clark et al., 2006; Park et al., 2006). These publications introduced the first evidence demonstrating PINK1 and Parkin presumably function in the same pathway to regulate mitochondrial function. Since then, the relationship between PINK1 and Parkin in regulating mitochondrial function has been a topic of great interest in the quest of understanding the interplay between these two genes in the context of neurodegeneration.

Mutations in the Parkin gene appeared to be responsible for autosomal recessive juvenile Parkinson's disease (Kitada et al., 1998). These mutations have been identified in nearly 50% of autosomal recessive and, 10 –15% of sporadic early onset PD. Parkin is a RING domain-containing E3 ubiquitin ligase involved in regulation of protein turnover by proteasomal degradation (Beasley et al., 2007). Parkin protein contains an N-terminal

ubiquitin-like (UBL) domain and 2 C-terminal RING finger domains that are separated by an in-between ring (IBR) domain (Beasley et al., 2007). As I will discuss later, the E3 ubiquitin ligase activity of Parkin is also involved in lysosomal degradation of damaged mitochondria by mitophagy.

In Drosophila melanogaster cells lacking Parkin or PINK1, the mitochondria are highly fused. This mitochondrial phenotype can be rescued upon over-expression of the GTPase protein, dynamin-related protein1 (Drp1), which regulates mitochondrial fission (Poole et al., 2008; Poole et al., 2010; Yang et al., 2008). Similarly, down regulation of mitochondrial fusion factors guanosine triphosphatase mitofusin/mitochondria assembly regulatory factor (Mfn/Marf) or Opa1 has been shown to induce the same compensatory effect (Poole et al., 2008; Poole et al., 2010). Based on the evidence, PINK1 and Parkin appeared to play a central role in regulating mitochondrial morphology and well being, but how did they interact to manage this process was unclear.

Interestingly, Narendra and colleagues provided exciting data indicating Parkin is selectively recruited to damaged mitochondria in the presence of the mitochondrial uncoupler, CCCP. This discovery was the first evidence introducing a link between mitochondrial quality control pathways and PD genes (Narendra et al., 2008). To this end, several studies regarding PINK1 and Parkin have emerged linking these proteins to the fission/fusion machinery, and the clearance of damaged mitochondria through a tightly regulated process such as mitophagy. Though the functional interaction between PINK1 and Parkin was demonstrated as early as 2006 (Clark et al., 2006; Park et al., 2006; Wang

et al., 2006), it was not until 2008 that PINK1-dependent phosphorylation of Parkin through direct binding of Parkin to PINK1 was established (Kim et al., 2008). Follow-up studies confirmed this regulatory function, and noted that PINK1-dependent phosphorylation of Parkin may play a role in mitochondrial quality control (Caulfield et al., 2014; Iguchi et al., 2013; Kazlauskaite et al., 2014; Kazlauskaite et al., 2015; Sha et al., 2010; Shiba-Fukushima et al., 2014a; Shiba-Fukushima et al., 2012; Shiba-Fukushima et al., 2014b).

In this regard, an exciting breakthrough in the field was made by Richard J. Youle laboratory, followed by many other reports, indicating that PINK1 and Parkin neurosurvival function is mediated by a mitochondrial quality control (MQC) pathway (Geisler et al., 2010; Matsuda et al., 2010; Narendra et al., 2010). The suggested model was particularly interesting since for a long time researchers in the field were puzzling over the nature of the relationship between PINK1, residing in inner mitochondrial membrane, and Parkin as a cytosolic protein. According to this model, in negatively charged mitochondria, PINK1 is cleaved by mitochondrial proteases such as MPP, PARL and AFG3L2 to produce multiple shorter cleaved products that are released to cytosol to be degraded by proteasomal activity (Greene et al., 2012). These PINK1 cleaved products are considered to be non-functional intermediates; a concept which is still under debate (Ivatt and Whitworth, 2014). One example that challenges this point of view is the neuroprotective role of cytosolic PINK1 against mitochondrial stressor, which has been reported by work in our lab and other laboratories (Dagda et al., 2014; Haque et al., 2008).

Nevertheless, proteolytic activity of PARL has been shown to target the PINK1 trans-membrane domain promoting re-distribution of PINK1 from mitochondrial inner membrane to cytosol or other mitochondrial compartment such as mitochondrial inter membrane space (Ivatt and Whitworth, 2014). Mitochondrial membrane depolarization prevents PARL proteolytic activity, leading to accumulation of full length PINK1 in mitochondrial outer membrane (Jin et al., 2010). PINK1 localization in the mitochondrial outer membrane leads to a configuration change causing its kinase domain to face toward cytosol where it recruits Parkin to damaged mitochondria through distinct steps (Jin et al., 2010). Recruitment of Parkin to mitochondria is believed to be involved with targeting of dysfunctional mitochondria and their isolation from the mitochondrial network to be degraded by autophagy (Geisler et al., 2010; Matsuda et al., 2010; Narendra et al., 2010). This process is disrupted by PD associated Parkin mutations (Geisler et al., 2010).

Relevant to this pathway, few downstream effectors such as ubiquitin, Mitofusins and Miro have been identified for PINK1 and Parkin (Chen and Dorn, 2013; Gegg et al., 2010; Koyano et al., 2014; Wang et al., 2011). For instance, in response to mitochondrial uncouplers such as CCCP, mitochondrial motility has been shown to be restricted via phosphorylation of Miro by PINK1 (Wang et al., 2011). Phosphorylated Miro is then ubiquitinated by Parkin triggering rapid degradation of Miro through the proteasome-dependent pathway (Wang et al., 2011). Similarly, PINK1 phosphorylates the mitochondrial outer membrane Mfn 2 to promote its ubiquitination in a Parkin-dependent manner. Ubiquitination of Mfn2 has been shown to activate cellular autophagic pathways directing damaged mitochondria for degradation (Chen and Dorn, 2013; Gegg et al., 2010).

Recently, a number of elegant studies have uncovered that ubiquitin is a target of PINK1 phosphorylation(Caulfield et al., 2014; Kane et al., 2014; Kazlauskaite et al., 2015; Koyano et al., 2014). Interestingly, this is the first evidence that shows ubiquitin is modulated by phosphorylation. As ubiquitination is a prominent feature of quality control of mitochondria, phosphorylation of ubiquitin has been studied in detail. For example, PINK1 has been shown to phosphorylate both ubiquitin and Parkin ubiquitin like domain (UBL) at serine 65 residue. In fact, PINK1-dependent phosphorylation of ubiquitin primes Parkin to bind to phosphorylated ubiquitin. Upon interaction with ubiquitin, Parkin undergoes a conformational change. The new Parkin configuration removes the inhibitory conformation of UBL domain. As a result, Parkin can be more efficiently phosphorylated by PINK1 at serine 65 residue of UBL, which leads to maximal activation of Parkin E3 ubiquitin ligase function (Kazlauskaite et al., 2015). However, as shown in **Figure 1.2**., the precise order of the events is still under debate. For instance, it has been proposed that Parkin' configuration is changed upon interaction with phosphorylated ubiquitin. This configuration exposes Parkin to PINK1 dependent phosphorylation. Upon phosphorylation by PINK1, Parkin more efficiently interacts with the ubiquitin chain to direct it to its specific substrates (Stolz and Dikic, 2014) **(Fig.1.2)**.

Although these findings have established one of the most attractive themes in the field, the relevance of this pathway or its downstream effectors in the pathogenesis of Parkinson's disease has yet to be proven.

Figure 1.2. PINK1, Parkin and quality control of mitochondria. ***Dysfunctional mitochondria:*** PINK1 is accumulated in outer membrane of dysfunctional mitochondria. PINK1 phosphorylates the ubiquitin-like domain (UBL) of Parkin on its S65 residue. Phosphorylation of Parkin leads to its conformational change, removing the auto inhibitory effect of UBL. PINK1 phosphorylates ubiquitin on its S65 residue. Interaction of phosphorylated ubiquitin and phosphorylated Parkin primes Parkin ubiquitin E3 ligase activity to more efficiently ubiquitinize its substrates on damaged mitochondria. ***Healthy mitochondria*****:** in normal healthy mitochondria, PINK1 is directed to mitochondrial inner membrane where it is cleaved by mitochondrial proteases to produce shorter products. PINK1 cleaved products are released to cytosol to be degraded by protosomal pathway.

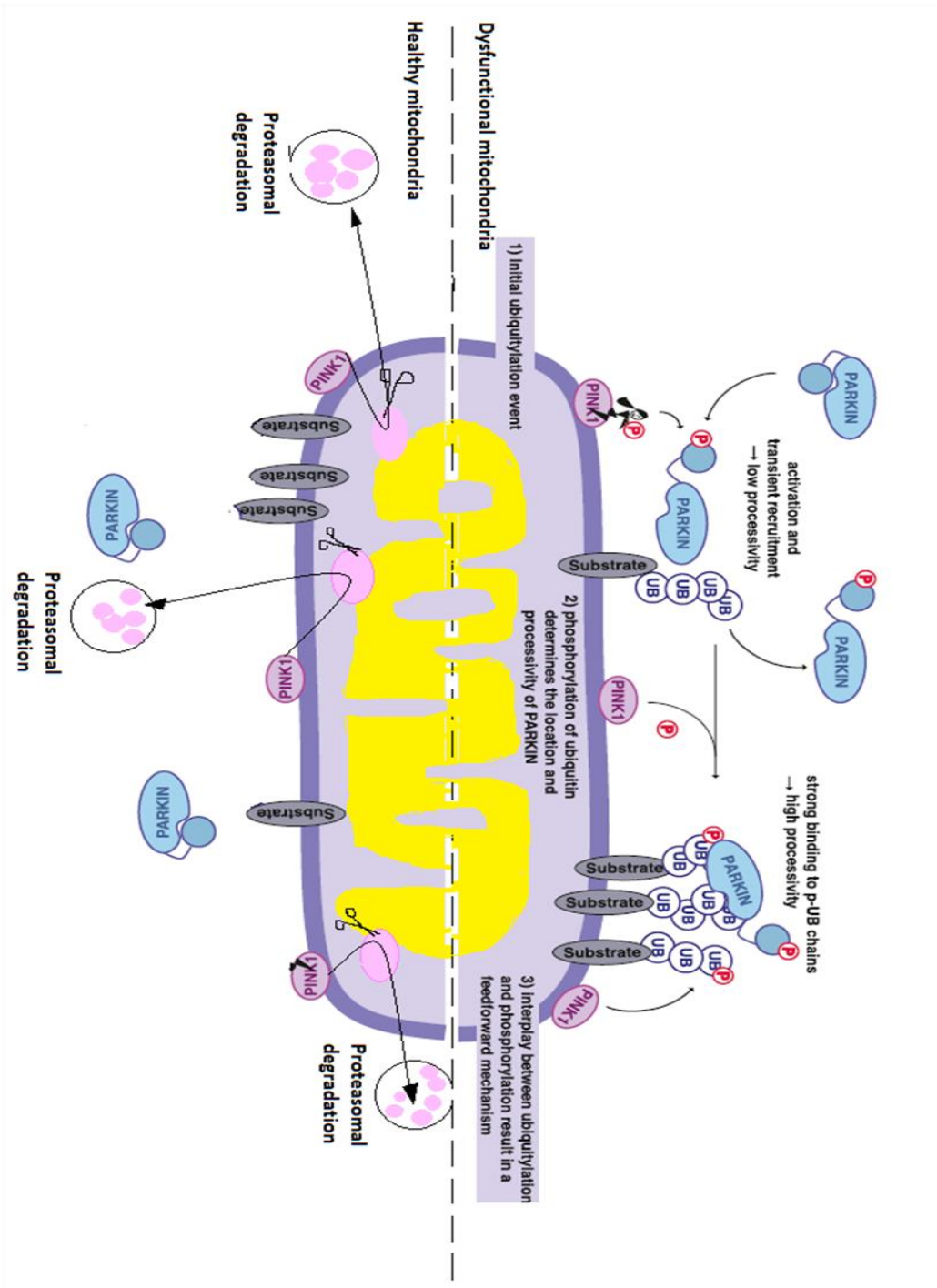

Healthy mitochondria
Dysfunctional mitochondria
1) Initial ubiquitylation event
2) phosphorylation of ubiquitin determines the location and processivity of PARKIN
3) interplay between ubiquitylation and phosphorylation result in a feedforward mechanism
activation and transient recruitment → low processivity
strong binding to p-UB chains → high processivity
PARKIN
PINK1
Substrate
UB
Proteasomal degradation

Figure 1.2

1.2.6. PINK1's pro-survival function in different animal models:

PINK1 mutations are rare causes of PD among familial cases, however, the presence of PINK1 mutations is considered a significant risk factor in sporadic cases, as well (Abou-Sleiman et al., 2006a; Choi et al., 2008; Kumazawa et al., 2008; Rogaeva et al., 2004). It is still unclear how PINK1 mutations contribute to the etiology of the disease although the neuroprotective role of PINK1 has been frequently confirmed in multiple experimental models of neurodegeneration. For instance, in Drosophila, PINK1 deficient flies display increased sensitivity to oxidative stress; abnormal mitochondrial phenotype, especially in testes and flight muscle, and progressive loss of dopaminergic neurons (Clark et al., 2006; Park et al., 2006; Yang et al., 2006). These phenotypes can be rescued by over-expression of human PINK1. Interestingly, antioxidant approaches such as expression of human SOD1 or administration of vitamin E prevent this dopaminergic neuronal loss (Wang et al., 2006). These findings further support the idea of a neuroprotective effect of PINK1 against oxidative stress, and support the notion that oxidative stress may play an important role in pathogenesis of PD. Furthermore, using human dopaminergic neurons and human neuroblastoma cell lines, *Wood-Kaczmar et. al.*,(2008) demonstrated that PINK1 is necessary for long-term survival and mitochondrial function in human dopaminergic neurons. They showed that over expression of wild-type PINK1, but not the Parkinsonism associated mutant, protects cells from apoptosis in response to oxidative stress.

The pro-survival effects of PINK1 are not limited to neuronal cells. It has been shown that the murine atrial cell line over-expressing PINK1 is more resistance against

simulated ischemia-reperfusion injury (Siddall et al., 2013). Additionally, myocardial infarct size is increased in PINK1 knockout (Ko) mice subjected to simulated ischemia-reperfusion injury (Siddall et al., 2013).

More relevant to our work, previous studies have established a role for PINK1 in protection against ischemic neuronal damage. For example, Chen et al. (2015) have shown that PINK1 deficiency sensitizes hippocampal CA1 neurons to transient global ischemia in rats. Using commercial antibodies against PINK1, they have also demonstrated that PINK1 is upregulated in hippocampal CA1 in response to transient global ischemia. They have also demonstrated that upregulation of PINK1 can enhance neuronal survival through down regulation of Drp-1 phosphorylation (Chen et al., 2015). Interestingly, these findings have hinted towards a pro-survival effect of PINK1 in ischemia context through regulation of mitochondrial morphology. However, data from our laboratory and others have confirmed that endogenous rat or mouse PINK1 is not detectable with the available commercial antibodies (Narendra et al., 2010; Zhou et al., 2008). Therefore, that makes it difficult to further interpret their data.

To further investigate the functional phenotype induced by PINK1 deficiency, Kitada and colleagues generated PINK1 null mice. These animals display reduced synaptic plasticity and impaired dopamine release in the striatum when compared to controls. However, the morphology and numbers of dopaminergic neurons in the substantia nigra are not affected by PINK1 deficiency (Kitada et al., 2007). This animal model has been extensively used in our laboratory in the present and previous studies. Our previous studies

have shown that PINK1 deficient dopaminergic neurons are more sensitive to the toxic effects induced by systemic MPTP (1-methyl-4- phenyl-1,2,3,6-tetrahydropyrindine) treatment, as an animal model of PD (Haque et al., 2012). However, the precise signalling pathways mediated by PINK1 to handle oxidative stress induced by MPTP, are still under investigation.

In response to oxidative stress, multiple intracellular mechanisms are activated to control the damage. Among them, the AKT (also Protein Kinase B, PKB) pathway most commonly responds to oxidative stress in a pro-survival manner (Vivanco and Sawyers, 2002). AKT is a serine/threonine kinase, which is involved in diverse cellular processes including regulation of apoptosis and metabolism, cellular proliferation, cell migration and metastasis (Sen et al., 2003; Vivanco and Sawyers, 2002). In human cells, AKT is present in three different isoforms: AKT-1, AKT-2, and AKT-3. In spite of structural similarities, they exhibit different patterns of tissue expressions. AKT1 protein contains 480 amino acids with an N-terminus pleckstrin homology (PH) domain. The PH domain mediates its interaction with membrane phospholipid, phophatidylinositol3 phosphate (PIP3). Activity of AKT is efficiently regulated through reciprocal phosphorylation and dephosphorylation of phosphatidylinositol by PI3K and PTEN, respectively. In fact, phosphorylation of phosphatidylinositol allows AKT to bind to PIP3 by its Pleckstrin Homology (PH) domain (Andjelkovic et al., 1997). This binding recruits AKT to the plasma membrane where it is phosphorylated by phosphatidylinositol dependent kinase 1 (PDK1) at threonine 308 (T308). However, full activation of AKT requires phosphorylation of another serine residue by the mTORC2 complex at serine 473 (S473) (Alessi et al., 1997). Once AKT is fully

activated it exerts its kinase function within the cell. A simplified diagram illustrates the pathway as following **(Fig.1.3).**

Figure 1.3: The PI3K-AKT-mTOR Pathway. A subset of AKT upstream regulators (including PI3K, PTEN, PDK and mTORC2) and downstream effectors are overviewed in this diagram.

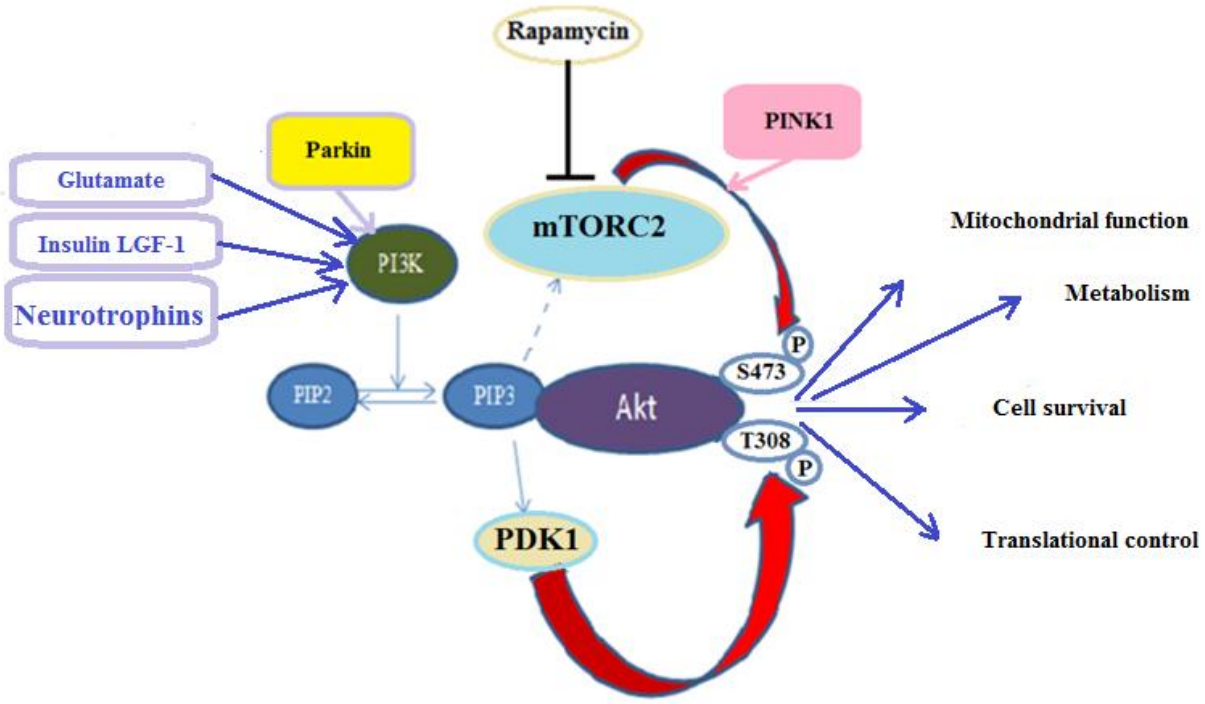

Rapamycin
PINK1
Parkin
Glutamate
Insulin LGF-1
Neurotrophins
PI3K
mTORC2
Mitochondrial function
Metabolism
P
S473
PIP2
PIP3
Akt
Cell survival
T308
P
PDK1
Translational control

Figure 1.3

Several lines of evidence suggest that AKT plays a neuroprotective role against ischemia (Ohba et al., 2004; Zhao et al., 2006a). For example, using the middle cerebral artery occlusion (MCAO) model of stroke, Ohba and colleagues have demonstrated that AKT overexpressing transgenic mice are more resistance against ischemia (Ohba et al., 2004). The neuroprotective effects of AKT are not limited to ischemia models. Previous studies, including work from our own lab, have established a neuroprotective role for AKT in PD (Aleyasin et al., 2010; Ries et al., 2006; Salinas et al., 2001).

Interestingly, it has been shown that Parkin deficiency accelerates endocytosis and degradation of epidermal growth factor receptor (EGFR) which reduces EGFR signalling via PI3K–AKT pathway (Fallon et al., 2006). The authors have proposed that reduction of PI3K–AKT signalling would increase susceptibility to neuronal death. Additionally, it has been shown that PINK1 enhances AKT phosphorylation (Murata et al., 2011). However, they have shown that PI3K signalling pathway is not involved in this enhancement. In fact, PINK1 contributes to phosphorylation of AKT via activation of mammalian target of rapamycin complex 2 (mTORC2). These findings support the idea of exploring whether pAKT mechanistically mediates PINK1 and Parkin neuroprotective function in ischemic model of neuronal injury.

1.3. Animal experimental models used, *in vivo* and *in vitro*

1.3.1. MCAO; a model of focal ischemia

Middle cerebral artery occlusion (MCAO) is the most commonly used animal stroke model to recapitulate human focal ischemia. This model was originally developed as an experimental stroke model in rats by Koizumi and colleagues in 1986. Later on, availability of mice transgenic lines has encouraged a great interest in mouse stroke models. Therefore, the experimental procedure for MCAO was extended to the mouse experimental model. This animal model has extensively been used to determine the mechanism of neuronal death in stroke, and to test novel therapeutic strategies. MCAO is induced by a number of surgical procedures including intraluminal suture model, photothrombosis, Endothelin-1, MCA clip or cautery, and embolism models (Carmichael, 2005).

In this study, MCAO has been achieved by intraluminal suture method. Briefly, an intraluminal suture has been inserted into the internal carotid artery via the external carotid artery to block the entry of the Middle cerebral artery. In this paradigm, depending on the duration of occlusion, MCAO induces ischemic neuronal death in striatum and overlying cortex including frontal, parietal, temporal, and occipital lobes. Additionally, MCAO could also induce ischemic damage in thalamus, substantia nigra and hypothalamus. Generally, MCAO in one hemisphere is associated with motor dysfunction and sensory loss in the contralateral side of the body **(Fig.1.4)**.

Figure 1.4. Schematic diagram illustrating intraluminal suture MCAO. (A) The left middle cerebral artery is occluded with a silicon-coated nylon suture, which is inserted into the left internal carotid artery (ICA) through the stalk of the external carotid artery (ECA). (B) Illustration of the middle cerebral artery supply area (O'Neill and Clemens, 2001).

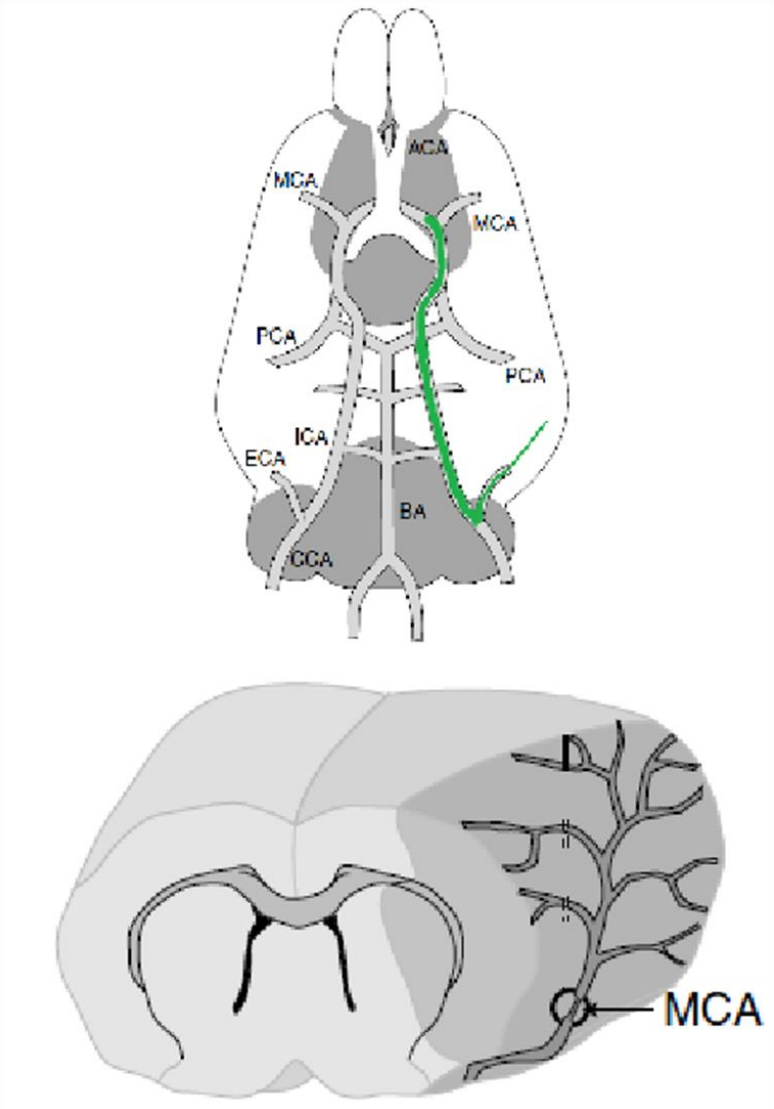


Figure 1.4

1.3.2. Glutamate treatment; an in vitro model of ischemia

Glutamate treatment has been widely used as an in vitro model to recapitulate the excitotoxicity and neuronal injury after ischemia (Arundine and Tymianski, 2004; Culmsee and Mattson, 2005; Culmsee et al., 2005). As mentioned earlier, a high long-lasting rise in the extracellular glutamate concentration induces neuronal death through excitotoxicity. The term"“Exitotoxicity"was first coined by Olney in 1969 to describe the toxic effect of excitatory amino acids, and their involvement in neuronal degeneration (Olney, 1969).

Glutamate-induced excitotoxicity is mainly mediated by NMDA receptors (NMDARs) (Choi, 1992). Excessive activation of NMDARs is highly implicated in neuronal death following acute brain injury such as brain ischemia, hypoxia and mechanical trauma (Arundine and Tymianski, 2003). Interestingly, chronic activation of NMDARs may also be implicated in the pathophysiology of chronic neurodegenerative diseases such as Parkinson`s disease, Alzheimer`s disease, Multiple sclerosis and Amyotrophic lateral sclerosis (ALS) (Kalia et al., 2008; Lipton, 2006).

Typically, NMDARs are heterotetramers containing two glycine-binding GluN1 subunits and two glutamate-binding GluN2 subunits. The GluN1 subunit is essential for the NMDARs to be functional. Structurally, NMDARs are composed of a heteromeric complex of GluN1 subunit and one of the four different GluN2 subunits (GluN2A-D) or two GluN3 subunits (GluN3A and GluN3B). Predominantly, NMDARs are expressed as a complex of GluN1 and GluN2B/ GluN2A. The three dimensional structure of the subunits is defined by the properties and the composition of the amino acids. Generally, the N terminus domains,

and the ligand binding domains face toward the extracellular space while the transmembrane domain spans the membrane to assemble the ion channel pore, and the carboxy terminal domain extends within the cytoplasm to convey the signal. The NMDAR are expressed in both pre-synaptic and post-synaptic membranes. They are modulated by a variety of compounds such as glutamate receptor blocker MK-801 and ketamine (Lee et al., 2014).

In the present study, glutamate treatment has been applied to induce excitotoxic neuronal death, thereby investigating the neuroprotective function of PINK1 in ischemia context.

1.3.3. CCCP treatment; to establish a model of mitochondrial quality control

Over last few years, extensive studies in mammalian cell lines have suggested that Parkin and PINK1 are involved in the quality control of mitochondria through mitophagic degradation of dysfunctional mitochondria. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) is a mitochondrial membrane uncoupler that has been widely used to induce mitochondrial depolarization. This synthetic compound acts as an ionophore in mitochondrial inner membrane. As a result, CCCP increases membrane permeability to H^+, which interferes with oxidative phosphorylation leading to mitochondrial depolarization (Geisler et al., 2010; Matsuda et al., 2010; Narendra et al., 2008; Narendra et al., 2010).

While CCCP has been proven to efficiently induce robust Parkin recruitment to damaged mitochondria in immortalized cells, reproducing the same data in neuronal

context appeared to be more challenging (Grenier et al., 2013). Recently, interesting work from our laboratory has demonstrated that in the absence of antioxidant supplement in the culture medium, CCCP efficiently induces Parkin recruitment to the mitochondria in cortical neurons (Joselin et al., 2012).

In the present study, I have successfully reproduced these data previously demonstrated by my colleagues, to establish a positive control for my own experiments.

1.4. Statement of research problem, rationale, objectives and hypothesis:

The field of stroke recovery has fallen short in terms of curing the disease since current standard treatments for stroke are mostly involved in mitigation of the signs and symptoms of the disease.

There is growing evidence suggesting that common mechanisms are involved in pathophysiology of multiple neurological diseases. In support of this concept, a novel study from our laboratory has established a key neuroprotective role for PD gene, DJ-1, in models of focal ischemia induced by cranial injection of Endothelin-1. The main idea of this study stems from the notion that DJ-1 is protective against oxidative stress, an important characteristic factor in the pathophysiology of ischemia (Aleyasin et al., 2007). Later on, we showed that the interplay of DJ-1 and PINK1 dynamically regulates mitochondrial morphology to promote survival against oxidative stress (Irrcher et al., 2010).

In light of our findings above, together with the knowledge that PINK1 is involved in the survival pathway against oxidative stress and mitochondrial dysfunction, we have sought to investigate if PINK1 is also implicated in survival pathways activated in an ischemic context.

In this regard, we also explored two major potential pathways to provide some mechanistic insight into how PINK1 may be protective. First, we aimed to investigate if Parkin and its role in mitochondrial quality control (Jin and Youle, 2013; Joselin et al., 2012; Koyano et al., 2014)) are implicated in the neuroprotective function of PINK1.

Previous studies from our lab have established a neuroprotective role for cytosolic PINK1 in a neurotoxin-model of cell death by MPTP, suggesting a putative cytosolic pathway for PINK1 signalling (Haque et al., 2012; Haque et al., 2008). Several studies, including work from our own lab, have linked PINK1 and Parkin as well as DJ-1 to the AKT pathway, a major neuronal pro-survival pathway induced upon oxidative stress (Aleyasin et al., 2010; Fallon et al., 2006; Murata et al., 2011). These findings prompted me to examine the pro-survival function of cytosolic PINK1 through a putative signalling pathway regulated by AKT.

Therefore, I hypothesized that ***PINK1 exerts a neuroprotective function through a mechanism distinct from the PINK1/Parkin mitochondrial quality control pathways via modulation of the AKT pathway.*** The elucidation of such a mechanism is the subject of chapter three of this thesis. I have aimed to address this hypothesis through a set of objectives.

Objective 1:

Determine if PINK1 is important in mediating neuronal survival after acute ischemic injury.

Objective 2:

Determine which functional domains of PINK1 are essential for its neuroprotective effect.

Objective 3:

Investigate if Parkin and mitochondrial quality control axis is involved in PINK1 neuroprotective effect.

Objective 4:

Determine whether PINK1 confers its neuroprotective function through AKT in a model of ischemia.

Chapter 2

Methodology

2. Methodology:

2.1. Mice

Germ line-deleted Parkin and PINK1 mice have been previously generated and described in detail (Itier et al., 2003; Kitada et al., 2007). All animal care and experimental procedures were approved by the University of Ottawa Animal Care Committee, and carried out in strict accordance with the Guidelines for the Use and Treatment of Animals presented by the Animal Care Council of Canada, and approved by the Canadian Institutes of Health Research.

2.2. Cerebellar granule neurons extraction:

Mouse cerebellar granule neurons were obtained from p7-9 mice. Briefly, mouse pups (p7-9) were decapitated. The skulls were cut off to expose the cerebellum. Cerebellums were cut from the rest of the brain and meninges were removed. Cerebellums were individually transferred to a petri dish (35 mm) containing 1ml dissecting solution (124 mM NaCl, 5.37 mM KCl, 1 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 14.5 mM D-(+)-Glucose, 25 mM HEPES, 3 mg/ml BSA, PH 7.4). The cerebellar tissues were broken down by pipetting up and down for few times. Then, the broken tissues were transferred to a falcon tube and spinned down at 1000 rpm, for 1 min at 4 °C to pellet the cells. The resulting pellet were incubated in 3 ml dissecting solution supplemented by 0.25 mg/ml trypsin (Sigma), at 37˚C for ~20 minutes. The trypsinization was terminated by adding 3 ml ice-cold dissection solution containing 0.2 mg/ml trypsin inhibitor (Roche) and 1 mg/ml DNaseI (Gibco). The suspensions were gently mixed by rocking the falcon tubes for 2

minutes. The dissected tissues were pelleted at 1000 rpm, for 1 min at 4 °C. The resulting pellets were gently suspended in 2 ml dissecting solution containing 2.7 mM $MgSO_4$ and 0.03 mM $CaCl_2$, by triturating with a flame polished Pasteur pipette, and were further incubated for 10 minutes. The top layer of the cells (~1-1.5 ml) was gently removed and transferred to a new tube. This step was repeated one more time after adding 2 ml dissecting solution containing 2.7 mM $MgSO_4$ and 0.03 mM $CaCl_2$ to the remaining cells. The top layers of cells collected during these final steps, were briefly centrifuged at 1000 rpm, for 1 min at 4 °C. The pellets were resuspended in EMEM media containing 10% dialyzed FBS (Gibco) 25 mM KCl, 2 mM glutamine (Gibco), 25 mM glucose and 0.1 mM gentamycin (sigma). The neurons were plated into 96 or 24 well plates at a density of approximately $8x10^4$ or $5x10^5$ cells/well, respectively. The plates were coated with poly-D-lysine (1mg/ml) prior to plating. The CGNs cultures were treated with the antimitotic Cytosine-1-β-D-arabinofuranoside (AraC, 10 μM final concentration; 18–24 hrs after plating; Sigma) solution, which kills dividing cells. AraC treatment reduces the amount of glia cells in the culture. AraC administered at this dose had no toxic effects on CGNs. Recombinant adenovirus expressing human E2F-1 or LacZ (multiplicity of infection (m.o.i.) of 6 or 10 as indicated) was added to cell suspensions immediately before plating. K^+ deprivation was performed 7 days after plating by washing each well twice with 750 μl of complete medium (final K^+concentration of 5 mm) and then incubating

For those experiments requiring protein over-expression, five-day plated CGNs were infected with recombinant adenoviruses expressing either GFP along with wild type PINK1, kinase dead PINK1 (K219M), a mutant of PINK1 associated with PD (G308D),

PINK1 with its mitochondrial localization sequence deleted (ΔN PINK1) or GFP by itself as control at appropriate multiplicity of infection. Cultured neurons were incubated at 37C˚, 5% CO2. 24 hrs after viral infection, expression of viral constructs was assessed by immune fluorescence microscope.

2.3. Primary cortical neurons:

Cortical neurons were obtained from E14-16 C57BL/6 wild type (WT) and knockout PINK1 and Parkin mice of either sex. Depending on the experimental procedures, the neurons were seeded in 96, 24 and 12 well plates at a density of approximately 80,000, 5×10^5 and 1×10^6 cell/well, respectively.

Briefly, pregnant mice were deeply anesthetized. Mice's uteruses were dissected out from visceral cavity through an abdominal incision. Embryos were removed from the uterus and transferred to Hanks' Balanced Salt Solution (HBSS) (modified without Calcium and Magnesium) (Hyclone, Gibco). Brains were carefully exposed by cutting the skull open. Brains were cut off from the base of the skull. Cerebral cortices were dissected off and meninges were removed. Cortices were individually transferred to an eppendorf tube containing 1ml HBSS supplemented with trypsin (0.25 mg/mL) (Sigma), incubated for 20 min at 37˚ C with agitation. Then, trypsinization was stopped by adding 500 µl ice-cold neurobasal medium supplemented by Trypsin inhibitor 0.2 mg/ml (Roche Diagnostics) and DNase1 (0.2 mg / ml) (Gibco). The tubes were gently turned upside down for 2 minutes by hand. Then, the tubes were spined down at 1000 x, for 5 min at 4 °C to pellet the cells. The supernatant medium was sucked out by a vacuum suction. The pellets were re-suspended in

500 µl ice-cold neurobasal supplemented by of trypsin inhibitor and DNase1. Cells were triturated by pipetting up and down, for few times. Viable cells were counted using sterile filtered trypan blue solution (0.4 %) (Sigma). Number of cells per ml was calculated by: cell count in the central square of hemocytometer x dilution factor x 10^4. Cells were diluted to desired concentration for culture, in Complete Neurobasal Medium containing: Neurobasal medium (Gibco, Life Technologies), B27 supplement (Invitrogen), N2 supplement (Invitrogen), L-Glutamine 200 mM (Gibco, Sigma) and 0.05 mg/ml Penicillin-Streptomycin. Primary cortical neuron cells were plated. Plates were coated with Poly-D-Lysine-Hydrobromide (1 mg/ml, sterile) (Sigma) prior to use. Poly-D-Lysine incubation was performed for at least 1 hr at 37˚ and then plates were washed. For immunostaining, sterilized coverslips were placed into 24 well culture dishes and then coated with poly-D-lysine. Cultures were incubated at 37 °C in a 5% CO2 atmosphere and fixed or lysed at indicated time points.

After plating, for long-term experiments, 50% of the cultured medium was changed with fresh medium every 5 days.

2.4. Survival assay:

Glutamate treatments were performed in the presence or absence of MK801. At the end point, neuronal cultures were either lysed or fixed to be assessed for nuclear integrity. Each treatment was performed in triplets, and the average for each treatment was calculated and recorded as the result.

Briefly, culture media were removed from the wells by vacuum suction. Then, the neuronal cultures in 96 well plates were lysed by adding 50 µl lysis buffer to each well. Neurons were suspended in the lysis buffer by pipetting up and down for few times. 10 µl of lysed cells were placed on a hemocytometer, and neurons were assessed for nuclear integrity under the microscope. The total number of intact nuclei per well treated with glutamate was evaluated and compared with the number of intact nuclei per control wells.

Alternatively, neuronal cultures infected with GFP expressing viruses were fixed with Lana's fixative, and stained with Hoechst 33258 (1:10000, Sigma-Aldrich B2883) for 30 minutes, for nuclei staining. Using fluorescent microscopy, GFP-positive neurons were explored for nuclear integrity. The total number of live GFP-positive neurons per well was evaluated and compared with the number of GFP-positive neurons in control wells.

2.5. Hypoxia:

For a more delayed model of death, 7 days-plated CGN cultures or 10 days-plated cortical neurons were incubated in hypoxia chamber (1% O2, 5% CO_2) in the presence of MK801 (10µM) for indicated time points, followed by re-oxygenation at 37ºC. Control plates contained MK801, but were not exposed to hypoxia. For a more excitotoxic model of death, infected cultures were incubated in the hypoxia chamber in the absence of MK801 for indicated time points followed by re-oxygenation. After each time course of re-oxygenation, neurons were fixed with Lana's fixative, and then stained with Hoechst 33342.

2.6. Glutamate treatment for survival assessment:

7 days-plated CGN neurons were exposed to glutamate treatment. Glutamate was added to the wells to a final concentration of 50 µM for 45 minutes, and then washed out with conditioned medium. Neuronal cultures were then incubated for 1 hr. This was in the presence or absence of MK801 (10µM). Conditioned medium was obtained from cultured neurons seeded in spare plates.

Alternatively, cortical neurons (10 days *in vitro*, DIV) were subjected to glutamate treatment (200µM) for 1 hr. Then, glutamate was washed out with conditioned medium. Neuronal cultures were subsequently incubated at 37C˚ for 1 hr. For neuronal cultures infected by adenoviruses, glutamate treatment was performed 2 days after infection, and for adeno-associated viral infection, glutamate treatment was performed 10 days after infection. Survival was evaluated as described above.

2.7. Detection of mitochondrial localization of Parkin after hypoxia, glutamate treatment or CCCP treatment:

For immunofluorescence assay of GFP-Parkin, neurons were seeded on PDL-coated cover slips in 24-well plates at a density of 3×10^5 cells per well. At the time of plating, AAV-viruses expressing GFP-fused wild type Parkin were introduced into primary cortical neuronal cultures of either wild type or knock out PINK1 mice. For a more delayed model of death, mitochondrial localization of Parkin was assessed following oxygen deprivation for 12, 18 or 24 hrs in presence of MK801 (10 µM), followed by 0, 12, 18 or 24 hrs re-

oxygenation. For a more excitotoxic model of death, mitochondrial localization of Parkin was assessed following oxygen deprivation for 1, 2, 3 or 4 hrs in the absence of MK801 followed by 0, 1, 2, 3 or 4 hrs re-oxygenation. Control plates contained MK801 but were not exposed to hypoxia.

For glutamate treatment, the time points were 0.5, 1 and 2 hrs Glutamate treatment (200 µM) followed by 0, 0.5, 1, 2, 3 or 4 hrs incubation after wash out with conditioned medium.

For CCCP treatment, cortical neurons were treated with 5 µM CCCP for 2 hrs. In all experimental paradigms, cultured media were changed to fresh media without B27 at least 24 hrs before experimental procedure. After treatment, cell cultures were fixed, permeabilized, and then stained with Hoechst 33342. Using an antibody against mitochondrial TOM20 complex, I assessed co-localization of the GFP-Parkin with TOM20 signal under confocal microscopy. Neurons displaying GFP-Parkin puncta were visualized and scored under fluorescence microscope.

2.8. Injection of Virus:

A midline sagital incision was made in the scalp of anaesthetized animals. Using a Hamilton syringe connected to syringe pump (PHD2000; Harvard Apparatus), viral constructs were introduced into the left dorsolateral striatum through a 1-mm burr hole (coordinates from bregma: anterioposterior 0.5 mm, lateral 3 mm, depth 3.5 mm). Viruses were injected during 12 min (0.25 µl/min).

2.9. Cerebral Artery Occlusion (MCAO):

Transient focal ischemia in 9-12 weeks old male mice was induced by MCAO. A midline neck incision was made. MCAO was achieved by introducing 9 mm of a silicone coated 7-0 monofilament suture into the MCA via the internal carotid artery. The monofilament was inserted to the internal carotid artery via an opening in stalk of the external carotid artery. After 30-60 minute MCAO the monofilament was withdrawn to permit reperfusion of the ischemic region. Body temperature was monitored with a rectal probe and maintained at 37ºC by a heating blanket. Animals were kept in an incubator, for 2 hrs following surgery.

For those experiments requiring protein over-expression, MCAO was performed 2 weeks after viral injection.

2.10. Laser-Doppler Flowmetry:

Local cerebral blood flow (LCBF) was monitored in the cerebral cortex of the left hemisphere in the supply territory of the middle cerebral artery (MCA) by Laser-Doppler Flowmetry. LCBF was measured before and 10 minute after occlusion. Blood flow was also measured after reperfusion.

2.11. Immunostaining

Neurons were seeded on glass coverslips and fixed in 4 % PFA in 1 x PBS, for 15 minutes. Fixed cells were permeabilized and blocked for 20 minutes at room temperature,

in 0.1 % Triton X-100, and 5 % BSA in 1x PBS, respectively. Coverslips were incubated with primary antibodies diluted in 5 % BSA in 1x PBS. The following antibodies were used in this study: rabbit anti-MAP2 (H-300) (1:500, polyclonal IgG, SantaCruz, sc-20172), rabbit anti-TOM20 (Santa Cruz, 1:500) for 1 hr, at room temperature. Coverslips were washed (3x) and incubated with FITC-secondary antibodies. Alexafluor 594 (1:1000) Goat-Anti-rabbit (Molecular Probes, A11037) were diluted in 5% BSA in 1x PBS, and then incubated with coverslips for 30 min, at room temperature.

To visualize intact nuclei, cells were stained with Hoechst 33258 (1:10000) (Sigma-Aldrich B2883) in 1x PBS, incubated for 30 min, at room temperature. Slides were immersed in distilled water to remove excess salt and then mounted on slides.

2.12. Histological Analysis:

Animals were humanely anesthetized with euthansol and perfused with 0.9% saline followed by 4% paraformaldehyde buffer (PFA, EMD, PX0055-3) in 1x PBS at pH=7.4. Then brains were removed, post-fixed in 4% paraformaldehyde for 24 hr. Consequently, brains were cryopreserved in 10% sucrose solution containing 0.2% sodium azide, over a 5-day course. The sucrose solution was changed twice per day. Brains were freezed by immerging them in cold Isopentane, for 1 minute. Isopentane was cooled down to -35˚C by dry ice before and over the procedure.

Fourteen micrometers coronal sections were obtained from these brains with aid of a cryostat. Sections were directly mounted onto Super Frost Plus microscopic slides 25 x 75 x 1.0 mm (Fisher brand, 12-550-15). In each collection, four sections were skipped in between each selection. The slides were stored between −20°C and −80°C until analysis. Brain slices were stained with Cresyl violet as described below.

2.13.Cresyl Violet Staining

Sections were thawed on slide warmer for 15-20 min, at 37 °C, and then placed in distilled water for 5 min. Sections were then stained with 0.2 % filtered cresyl violet acetate ($C_{18}H_{15}N_3O_3$, Sigma C5042-10G, pH=3.5), for 10-30 minutes depending on the stain concentration. Sections were then dehydrated in graded ethanol solutions (50 %, 70%, 90% and 100 %) followed by defatting with xylene. Sections were mounted with coverslips using toluene.

Live tissue in supply area of Middle Cerebral Artery (Striatum, hippocampus and cortex) were considered as a cellular region with clearly intact nuclei and round soma. Neurons in dead or infarct area were stained as shrunken eosinphilic inclusion with pyknotic nuclei. Computer-controlled microscopic images were captured, and infarct volume was measured using Northern Eclipse software.

2. 14. Antibodies and Western blot analysis:

Primary cortical neurons were seeded in 12-well plates, and infected with AAV-viruses expressing GFP, GFP-PINK1 or GFP-Parkin. At the indicated experimental time points, the total cell lysates were collected and subjected to Western blot analysis.

Briefly, protein extracts were resolved on a 10% SDS - polyacrylamide gel, along with Molecular Marker, Blueye Prestained Protein Ladder (GeneDirex, P14007-0500). Transfers were done on 0.45 μM PolyVinylidene DiFluoride (PVDF) Transfer membrane (Immobilon-P, IPVH00010). Membrane was blocked in 5 % Bovine Serum Albumin (BSA) fraction V (Fisher, BP1600-100) in 1 x PBST [Phosphate Buffered Saline (pH=8.0) and 0.1 % Tween] at room temperature, for 1 hr. Proteins were detected using antibodies. Primary antibodies were diluted in 3 % Bovine Serum Albumin (BSA) fraction V (Fisher, BP1600-100) in PBST and 0.1 % Sodium Azide NaN3 (Sigma Ultra, s-8032) and incubated with the membranes overnight, at 4 °C, with agitation. Membranes were washed with 1 x PBST (3x) and then incubated with secondary antibodies diluted in 5 % non-fat milk, for 1 hr, at room temperature. Membranes were washed with 1 x PBST (3x). Immunoreactivity was detected using chemiluminescence HRP substrate [(Immobilin Western Chemiluminescence HRP substrate, Millipore, WBKLS0500) or (Pierce ECL Western Blotting Substrate, Thermo Scientific, 32106)] and visualized by autoradiography film (HyBlot CL Autoradiography film, Denville Scientific). Protein levels were normalized against β-actin signals, and results were reported in reference to control values.

The level of over-expressed PINK1 or Parkin, was assessed by immunobloting using PINK1 or Parkin antibody. For endogenous AKT or pAKT, the total cell lysates were collected after glutamate treatment, and analyzed by Western blot. The following antibodies were used in this study: rabbit anti-PINK1 (Novus, 1:5000), mouse anti-Actin (Sigma, 1:50,000), rabbit anti-Parkin (Millipore, 1:1000), Rabbit anti-AKT (Cell signaling,

1:5000), Rabbit anti-pAKT (Cell signaling, 1:2500), anti-mouse and anti-rabbit horse radish peroxidase-conjugated secondary antibody (Thermo, 1:10,000).

2.15. Quantification of Signal Density on Images

Immunoblots were further analyzed by Image J software to compare the density/intensity of the western blot membranes. Following imaging of the blots, representative bands were selected and their intensity was quantified. Then quantified measurements were transferred to excel and results were normalized against relative controls.

2.16. Rapamycin treatment:

Primary cortical neurons were seeded in 96 well plates. At the time of plating, neuronal cultures were infected with AAV-viruses expressing GFP-PINK1, GFP-parkin or GFP. Eight days after plating, neuronal cultures were treated with Rapamycin (1µM) or its vehicle, DMSO. Two days later, the neuronal cultures were subjected to glutamate treatment. Survival assay was performed as described above.

2.17. LY294002 treatment:

Primary cortical neurons were seeded in 96 well plates. At the time of plating, neuronal cultures were infected with AAV-viruses expressing GFP-PINK1, GFP-parkin or GFP. Ten days after plating, neuronal cultures were treated with LY294002 (10µM) or its vehicle, DMSO. One hr later, neuronal cultures were subjected to glutamate treatment. Survival assay was performed as described above.

2.18. Statistical analysis:

Data subjected to multiple comparisons using ANOVA. Student's t-test was applied for two group comparisons. All data were presented as mean ± standard error of the mean (SEM). Significance at *P < 0.05, **P < 0.01 and ***P < 0.001; no significance is denoted by n.s.

Chapter 3

Results

3. Results

In chapter 1, I reviewed the literature surrounding the thesis topic including the current body of evidence related to stroke and Parkinson's disease (PD) research. I further provided background knowledge supporting the rationale for this doctoral work. In the following sections, I have described the main findings of this thesis. In each section, I present the results by stating the rationale, a brief description of the approaches taken to achieve the results, and a brief conclusion.

3.1. Characterization of a novel pro-survival role for PINK1 in response to ischemia-induced neuronal injury

3.1.1. PINK1 null neurons are more sensitive to excitotoxicity in model of death in vitro.

PINK1 deficiency leads to decrease of the mitochondrial calcium capacity. This lowers the threshold of opening of the mitochondrial permeability transition pore (mPTP) making neurons more vulnerable to death (Gandhi et al., 2009). However, the effect of PINK1 deficiency in neuronal death after more acute events such as ischemia is not clear. To directly address these issues, and test my hypothesis that PINK1 is involved in neuronal survival following stroke injury, we employed glutamate treatment in multiple cell types including CGNs and primary cortical neurons as an *in vitro* model of ischemia. To eliminate glial cells, the CGNs cultures were treated with AraC. Moreover, serum-free Neurobasal medium, support differential growth of primary cortical neurons obtained from cerebral cortex. In these conditions, glial growth was reduced to less than 0.5% of the

nearly pure neuronal population which excludes the global effects of PINK1 on the non-neuronal cells from our study. Consequently, neuronal survival rate was assessed as described in *Methodology*. As shown in **Figure 3.1**, PINK1-null Cerebellar Granular Neurons (CGNs) are significantly more sensitive to glutamate treatment.

Figure 3.1. PINK1 deficiency increases sensitivity of CGNs to glutamate-induced cytotoxicity. CGNs were harvested from 7-9 days old PINK1 knock out (n=6) and wild type (n=6) pups. Seven days after plating, neurons were transiently treated with 50 µM glutamate in either the presence or absence of 10 µM MK801 for 45 minutes, followed by 60 minutes incubation period. Non-treated neurons were used as the control group to determine the survival rate. (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$)

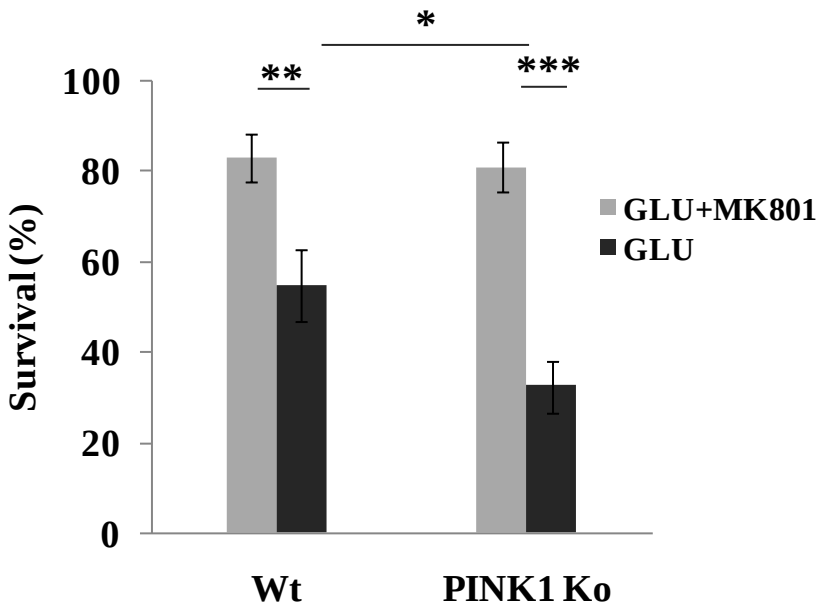
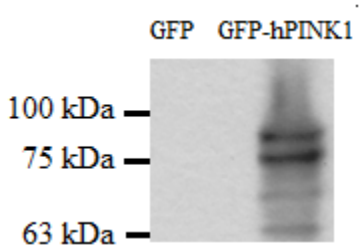


Figure 3.1

Later, to confirm if the sensitivity observed in PINK1-null neurons is specifically induced by PINK1 deficiency, we expressed GFP alone or GFP-tagged wild-type human PINK1 (hPINK1) in the CGNs of wild-type and PINK1 knockout pups. Consistent with the previous result, we showed that the wild-type neurons infected with wild-type hPINK1 were significantly more resistant to excitotoxicity induced by glutamate treatment. Furthermore, over-expression of hPINK1 rescued the neuronal sensitivity induced by PINK1 deficiency **(Fig. 3.2)**. We have also examined if cortical neurons of PINK1 null mice are more sensitive to glutamate-induced neuronal damage. Interestingly, our data indicated that the effect of PINK1 deficiency was not restricted to CGNs, as it affects cortical neurons as well **(Fig. 3.3)**.

Figure 3.2. Over-expression of wild type hPINK1 protects CGNs against glutamate-induced cytotoxicity. Five days after plating, CGNs, harvested from 7-9 days old wild type and PINK1-null pups were infected by rAV vectors carrying WT GFP-hPINK1 construct along with GFP. **(A)** Expression of viral constructs in CGNs was shown by Western blot analysis. **(B)** Forty eight hrs after viral infection, neurons were transiently treated with 50 µM glutamate in the presence and absence of 10 µM MK801 for 45 minutes, followed by 60 minutes incubation period. Non-treated neurons were used as the control group to determine the survival rate. (n=4, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$)

(A)


GFP
GFP-hPINK1
100 kDa
75 kDa
63 kDa

(B)

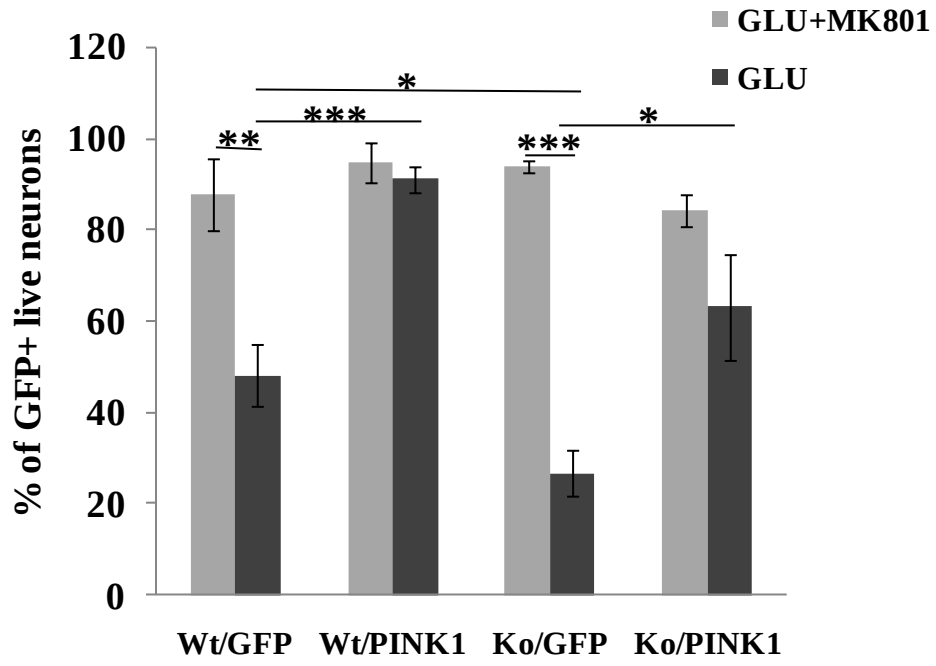

GLU+MK801
GLU
% of GFP+ live neurons
120
100
80
60
40
20
0
*
***
**
***
*
Wt/GFP
Wt/PINK1
Ko/GFP
Ko/PINK1

Figure 3.2

Figure 3.3. PINK1 deficiency increases sensitivity of cortical neurons to glutamate-induced cytotoxicity. Cortical neurons were harvested from 14-16 days old PINK1 knock out (n=7) and wild type (n=8) embryos. Ten days after plating, neurons were transiently treated with 200 µM glutamate in the presence and absence of 15 µM MK801 for 60 minutes, followed by 60 minutes incubation period. Non-treated neurons were used as the control group to determine the survival rate. (** $p<0.01$ and *** $p<0.001$)

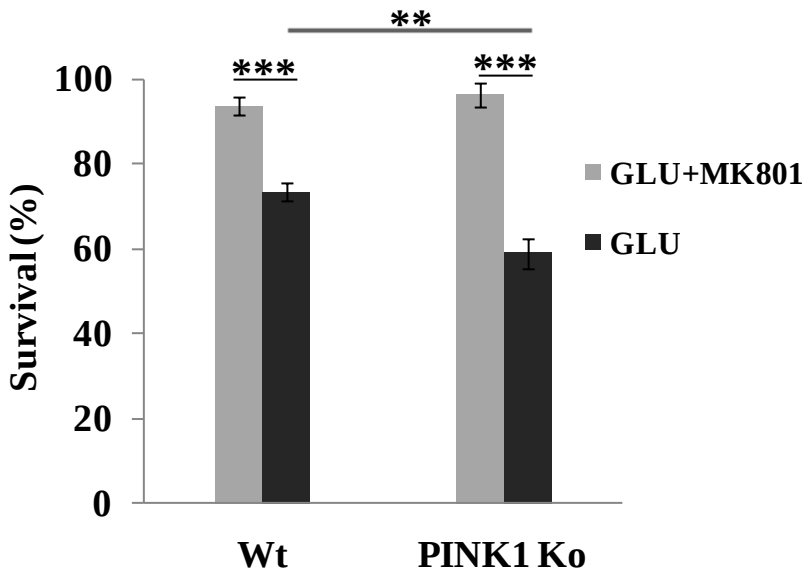


Figure 3.3

3.1.2. PINK1 null mice are more sensitive to brain damage induced by MCAO as in-vivo model of ischemia.

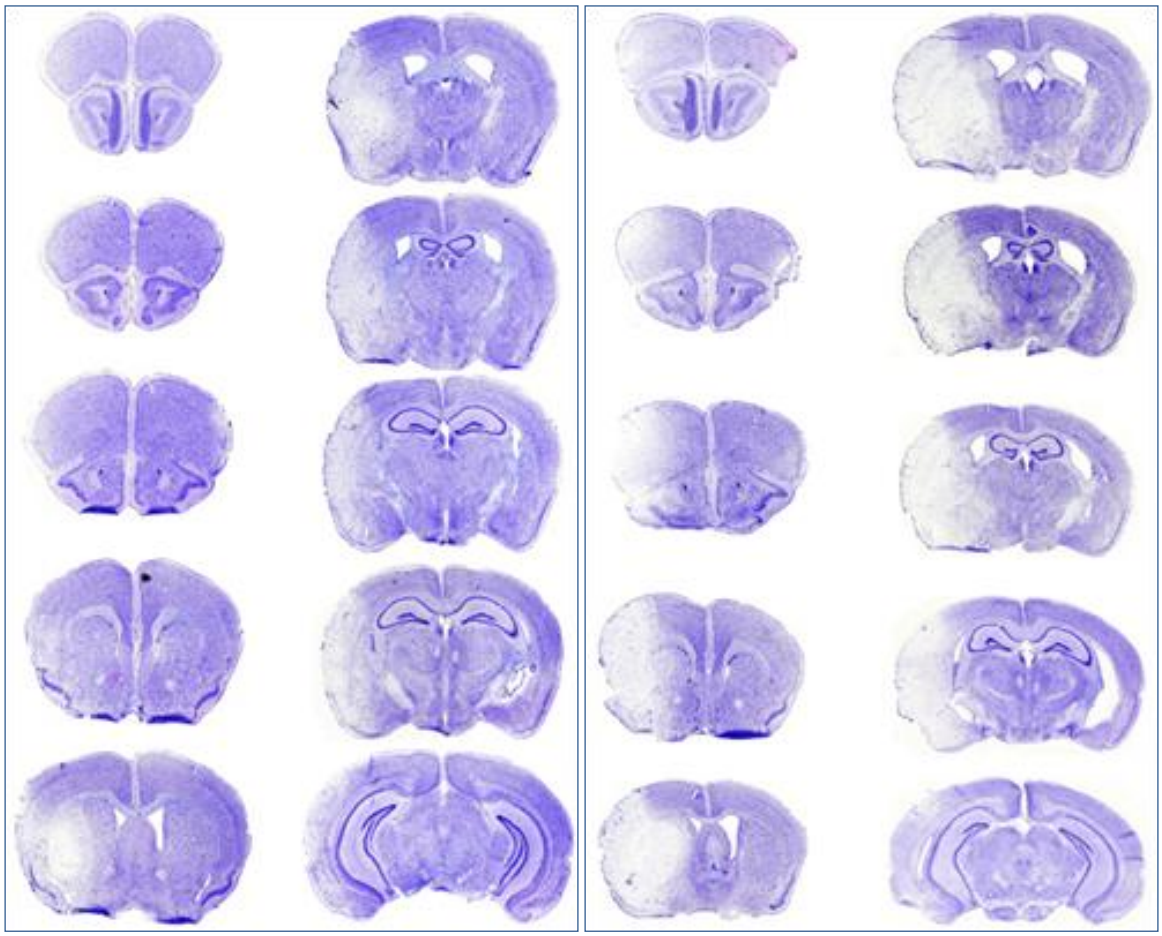
To validate our *in-vitro* results, and to test our hypothesis in a more clinically relevant paradigm, we examined whether PINK1 deficient mice are more susceptible to ischemia-induced brain damage *in vivo*. To achieve this, we performed transient MCAO (30 min) as a model of ischemia/reperfusion. Seventy-two hrs after reperfusion, animals were sacrificed and prepared for histological analysis. To assess stroke-induced neuronal damage, we first stained coronal brain sections for cresyl violet to visualize the infarct lesion **(Fig. 3.4A)**. As described in M*ethodology*, infarct volume was calculated by assessing sequential brain slices for infarct lesion. Consistent with our *in vitro* observations, PINK1 deficient animals showed larger infarct volume in response to MCAO when compared to the wild type animals **(Fig. 3.4B)**. Together, these findings indicate that PINK1 has a critical role in neuronal response to ischemic insult. However, it is not clear which functional or structural attributes of PINK1 are required for its pro-survival role activated in response to ischemic injury.

Figure 3.4. PINK1 deficiency increases sensitivity of brain tissue to 30 minute acute focal ischemia. (A)

Representative pictures of Cresyl violet staining of 14 µm thick brain slices of WT mice (n=10) and PINK1 knockout mice (n=6) after 30 minutes MCAO followed by 72 hrs reperfusion. (B) Quantification of the infarct volume in the brain slices (* $p<0.05$).

(A)

Wild type **PINK1 Knockout**



(B)

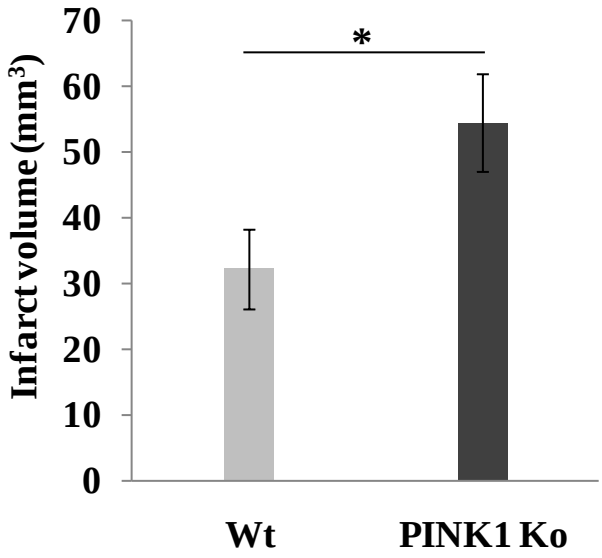


Figure 3.4

3.1.3. Intact kinase activity is required for PINK1 neuroprotective function in cytosol.

The N-terminal mitochondrial targeting motif and the kinase domain are two characteristic functional domains of PINK1. As already described, it has been shown that the kinase activity of PINK1 is important for its pro-survival function (Petit et al., 2005; Sim et al., 2006). Additionally, mitochondrial localization of PINK1 has been proposed to be important for its neuroprotective function following CCCP treatment (Joselin et al., 2012; Matsuda et al., 2010; Narendra et al., 2010). However, it is unclear whether the kinase activity of PINK1 or its mitochondrial localization is central to its pro-survival effect in a model of stroke. Therefore, to examine which domain exerts PINK1 neuroprotective effect against glutamate treatment, I over-expressed wild type hPINK1 along with different mutant forms of PINK1 including G309D, K219M and PINK1 with deleted mitochondrial motif (ΔN) in 5DIV CGNs.

G309D is one of the PINK1 mutants related to familial Parkinson's disease. This mutant has shown significant reduction in its kinase activity (Petit et al., 2005). Consistent with previous studies (Haque et al., 2008; Petit et al., 2005), our results show that this catalytic mutation abrogated PINK1 neuroprotective effect against glutamate-induced excitotoxicity **(Fig. 3.5)**. Equally, the kinase dead mutant, K219M, also lead to impaired neuroprotective effect. These findings additively support the important role of kinase activity of PINK1 in its neuroprotective function in our model. To examine if mitochondrial localization of PINK1 is important for its neuro-protective function, we also induced a

PINK1 mutant with deleted mitochondrial targeting motif (ΔN). This PINK1 mutant lacks the first 111 amino acids from the N terminus. Interestingly, we found that this mutant still robustly protects against glutamate toxicity **(Fig. 3.5)**. The latter finding suggests that PINK1 mitochondrial localization is not necessary for its neuroprotective function in these experimental settings.

These findings, in line with our previous studies, strongly support the idea that a potential cytoplasmic pathway is involved in the neuroprotective function of PINK1 (Haque et al., 2008).

Figure 3.5. Elimination of kinase activity significantly reduces the neuroprotective effects of PINK1. Five days after plating, CGNs harvested from 7-9 days old WT pups were targeted by rAV vectors carrying indicated constructs along with GFP. Forty-eight hrs after viral infection, neurons were transiently treated with 50 µM glutamate in the presence and absence of 10 µM MK801 for 45 minutes, followed by 60 minutes incubation. Non-treated neurons were used as the control group to determine the survival rate. (n=4, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

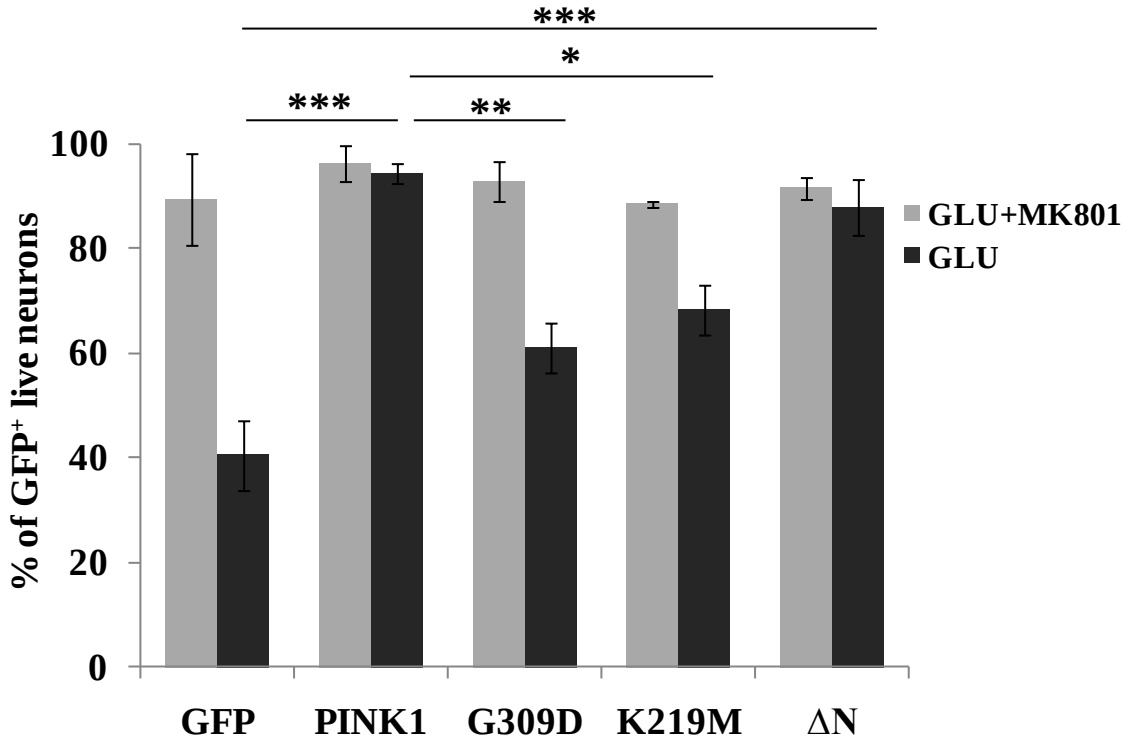

***
*
***
**
100
80
60
40
20
0
% of GFP+ live neurons
GLU+MK801
GLU
GFP
PINK1
G309D
K219M
ΔN

Figure 3.5

3.1.4. Over-expression of human Parkin partially rescued neuronal sensitivity induced by PINK1 deficiency in a model of ischemia.

The link between PINK1 and Parkin in regulating mitochondrial function in the context of neurodegeneration has been a topic of great interest to many laboratories including our own (Geisler et al., 2010; Joselin et al., 2012; Matsuda et al., 2010; Narendra et al., 2010).

In light of these studies, we sought to determine the role of Parkin as a potential mediator of PINK1 neuroprotective function. To do so, we first treated WT and Parkin null CGNs with glutamate. As shown in **Fig. 3.6**, Parkin null CGNs neurons were more vulnerable to stress induced by glutamate treatment. Likewise, Parkin null cortical neurons were more sensitive to glutamate treatment when compared to wild type control **(Fig.3.7)**. These findings are particularly interesting as this is the first report demonstrating Parkin is protective against excitotoxicity.

Figure 3.6. Parkin deficiency increases sensitivity of CGNs to glutamate-induced cytotoxicity. CGNs were harvested from 7-9 days old Parkin knock out (n=5) and wild type (n=6) pups. Seven days after plating, neurons were transiently treated with 50 µM glutamate in the presence and absence of 10 µM MK801 for 45 minutes, followed by 60 minutes incubation period. Non-treated neurons were used as the control group to determine the survival rate. (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$)

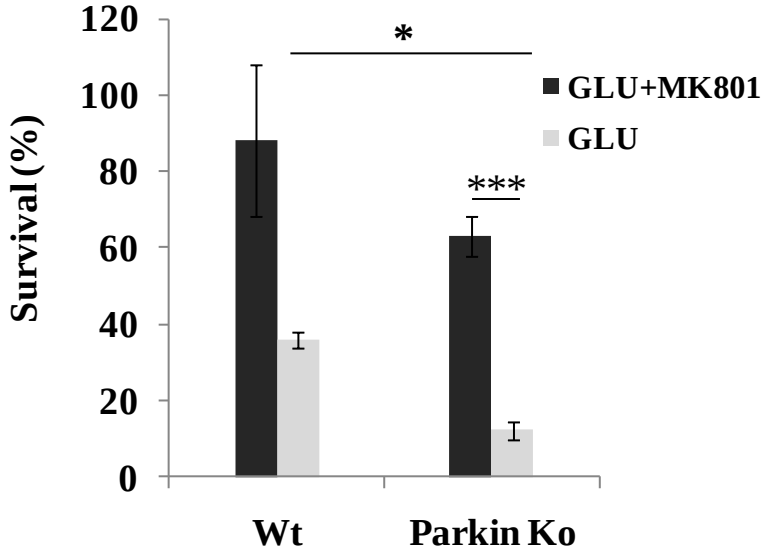


Figure 3.6

Figure 3.7. Parkin deficiency increases sensitivity of cortical neurons to glutamate-induced cytotoxicity. Cortical neurons were harvested from 14-16 days old Parkin knock out (n=7) and wild type (n=8) embryos. Ten days after plating, neurons were transiently treated with 200 µM glutamate in the presence and absence of 15 µM MK801 for 60 minutes, followed by 60 minutes incubation period. Non-treated neurons were used as the control group to determine the survival rate. (* $p<0.05$ and *** $p<0.001$).

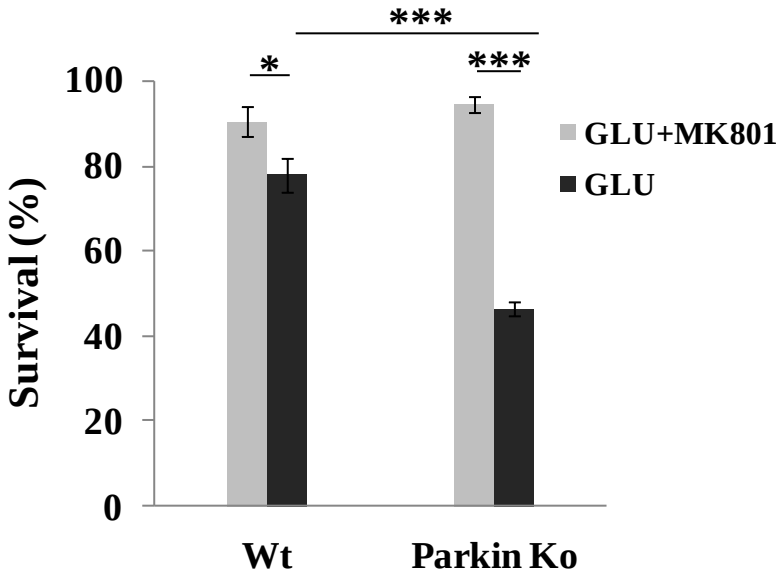


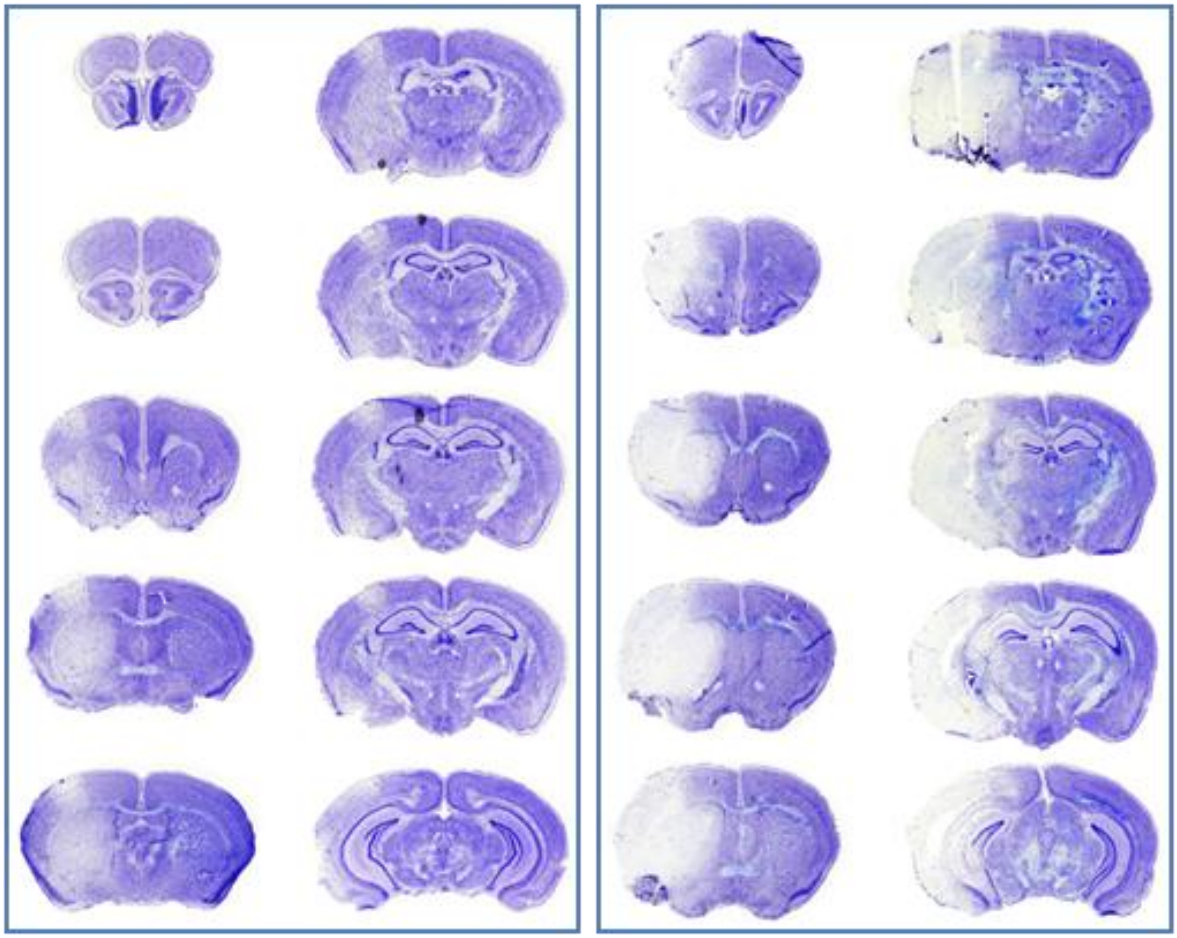
Figure 3.7

To confirm these results *in vivo*, we examined whether Parkin deficient mice are more susceptible to ischemia-induced brain damage. To achieve this, we performed transient MCAO (30 min) as a model of ischemia/reperfusion. Consistent with our *in-vitro* observations, 30 minutes MCAO induced a significantly bigger infarct lesion in the brain tissue of Parkin deficient mice **(Fig.3.8)**.

Figure 3.8. Parkin deficiency increases sensitivity of the brain to 30 minute acute focal ischemia.(A) Representative pictures of the Cresyl violet staining of 14 µm thick brain slices of WT mice (n=6) and Parkin knockout mice (n=6) after 30 minutes MCAO followed by 72 hrs reperfusion **(B)** Quantification of the infarct volume using the brain slices (* $p<0.05$).

(A)

Wild type **Parkin Knockout**



(B)

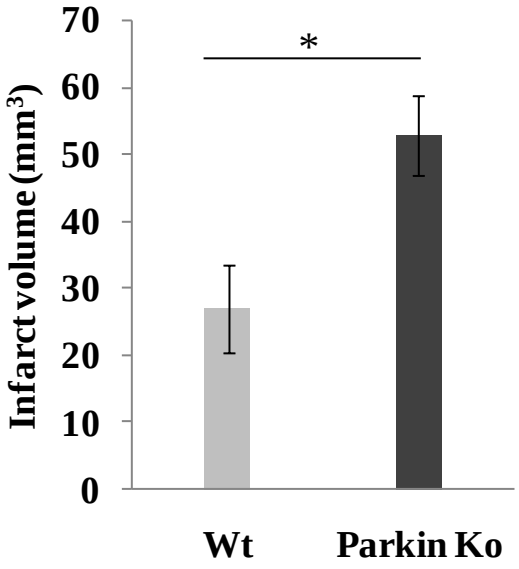
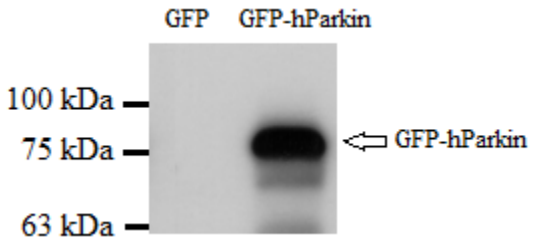


Figure 3.8

We next investigated if Parkin functionally interacts with PINK1, or whether Parkin and PINK1 function through different pathways. Using recombinant adeno-associated viral vectors (rAAV), we expressed GFP alone or GFP-fused wild-type hParkin in WT and PINK1 knockout postnatal CGNs. Seven days later, we treated CGNs with glutamate as described in *Methodology*. Interestingly, hParkin was capable of partially rescuing PINK1 loss mediated sensitivity **(Fig.3.9)**. To examine if PINK1 could rescue Parkin deficiency, in the reverse experiment, we over-expressed GFP alone or GFP-fused wild-type hPINK1 in the WT and Parkin knockout CGNs (5DIV). Two days later, we treated CGN cultures with glutamate as described. Interestingly, our data indicates that neuroprotective effect of PINK1 was aberrant in the absence of Parkin **(Fig.3.10)**.

Figure 3.9. Over-expression of hParkin protects wild type and PINK1 deficient CGNs against glutamate-induced cytotoxicity. At the time of plating, CGNs, harvested from 7-9 days old WT or PINK1 deficient pups, were infected by rAAV vectors carrying WT GFP-hParkin construct along with GFP. **(A)** Expression of constructs in CGNs was shown by Western blot analysis. **(B)** Seven days after viral infection, neurons were transiently treated with 50 µM glutamate in the presence and absence of 10µM MK801 for 45 minutes, followed by 60 minutes incubation period. Non-treated neurons were used as the control group to determine the survival rate. (n=3, ** $p<0.01$ and *** $p<0.001$)

(A)


GFP
GFP-hParkin
100 kDa
75 kDa
63 kDa
GFP-hParkin

(B)

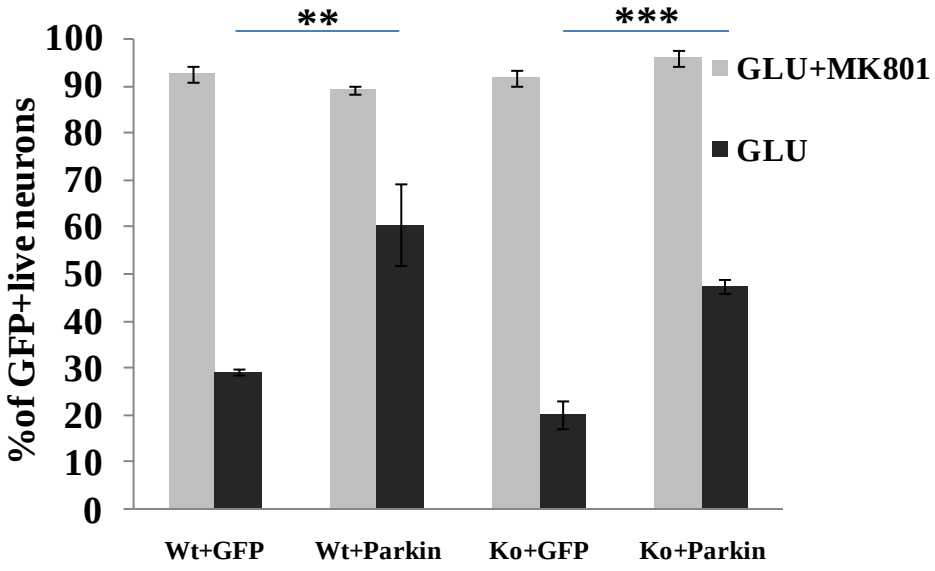

**
***
100
90
80
70
60
50
40
30
20
10
0
%of GFP+live neurons
GLU+MK801
GLU
Wt+GFP
Wt+Parkin
Ko+GFP
Ko+Parkin

Figure 3.9

Figure 3.10. Neuroprotective effect of over-expressed hPINK1 is reduced but not absent in Parkin deficient CGNs. CGNs, harvested from 7-9 days old WT and Parkin knock out pups (5DIV), were infected by rAV vectors carrying WT hPINK1 construct along with GFP. Two days after viral infection, neurons were transiently treated with 50 µM glutamate in the presence and absence of 10µM MK801 for 45 minutes, followed by 60 minutes incubation period. Non-treated neurons were used as the control group to determine the survival rate. (n=3, ** $p<0.01$ and *** $p<0.001$)

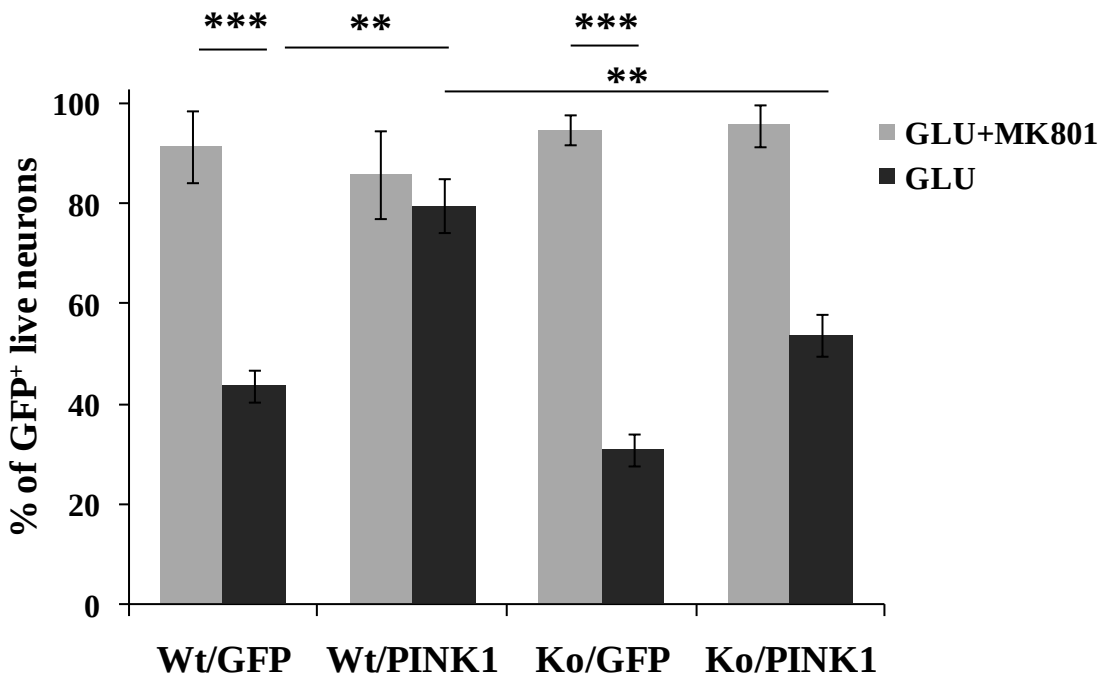

***
**
***
**
100
80
60
40
20
0
% of GFP+ live neurons
GLU+MK801
GLU
Wt/GFP
Wt/PINK1
Ko/GFP
Ko/PINK1

Figure 3.10

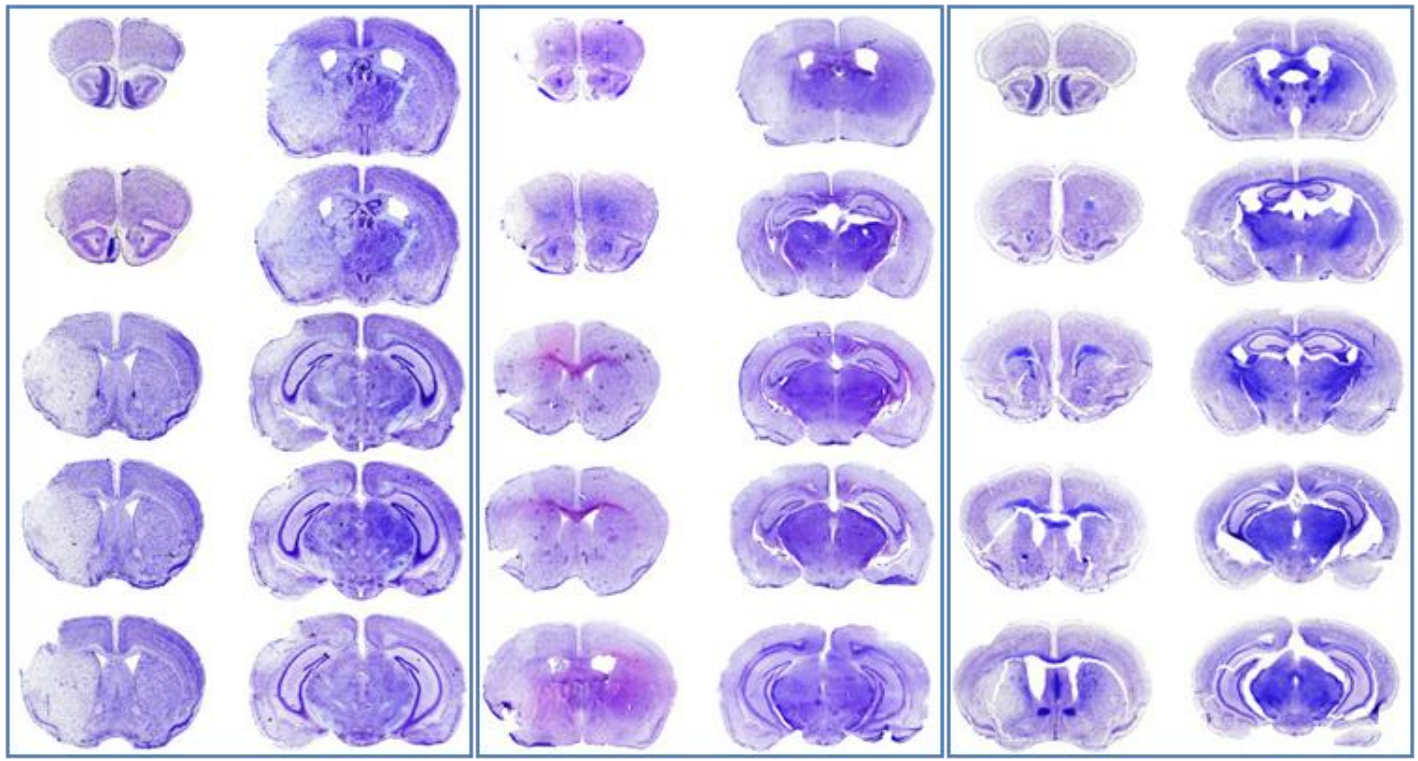
3.1.5. Over-expression of hParkin protects the brain tissue against ischemia and reverses the sensitivity induced by PINK1 deficiency.

The above evidence demonstrates that Parkin is important in cellular response to disturbance of calcium homeostasis in an *in vitro* model of ischemia. To further support this observation, and to examine this response in a more clinically relevant model of stroke, we examined whether induction of WT hParkin can protect the brain against ischemia *in-vivo*. To achieve this, we injected adeno-associated viral (AAV) vectors harbouring GFP-fused with wild type hParkin or GFP alone into the striatum of WT or PINK1 deficient mice. The use of AAV has proven to be useful in obtaining effective neuron- specific, genetic modulation in a relatively short period of time (2-4 weeks). This approach in our knockout system excludes the global effects of PINK1 deficiency on the non-neuronal cells from our study. Two weeks after virus injection, we performed a transient MCAO surgery for 20 minutes. Seventy two hrs after reperfusion, animals were sacrificed and prepared for histological analysis. As described before, infarct volume was assessed using the sequential coronal brain sections stained for cresyl violet **(Fig. 3.11A & Fig. 3.11B)**. As indicated in **Figure 3.11**, histological analysis of brain tissues revealed that over-expression of hPINK1 or hParkin in the striatum of WT mice could significantly reduce the infarct volume, compared to GFP expressing control mice **(Fig. 3.11D)**. Interestingly, over-expression of hParkin in the striatum of PINK1 knockout mice has also significantly reduced the infarct volume, compared to GFP expressing control mice **(Fig. 3.11D)**. There was no significant difference between the neuroprotective effect of PINK1 and Parkin either in WT or PINK1-null mice brains.

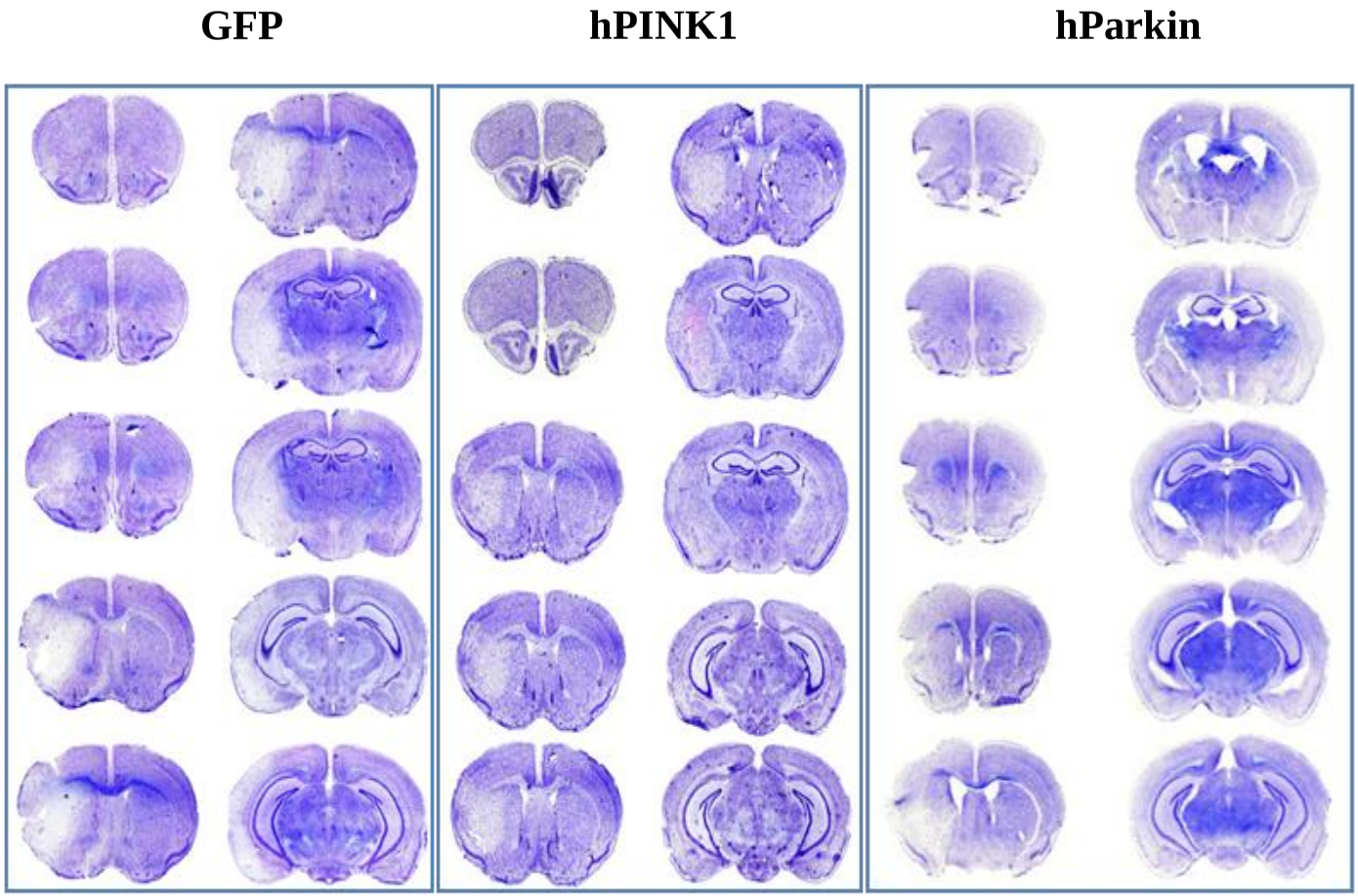
Figure 3.11. Over-expression of hPINK1 or hParkin in striatum of wild type mice protects brain tissues against stroke furthermore, over-expression of hPINK1 or hParkin in striatum of PINK1 knockout mice rescues hypersensitivity of brain tissues against stroke. Representative pictures of the Cresyl violet staining of 14 µm thick brain slices of **(A)** WT and **(B)** Knockout mice after 20 minutes MCAO followed by 72 hrs reperfusion. Wild type mice received stereotaxic injection of AA-viruses expressing GFP (n=7), hPINK1 (n=5) or hParkin (n=5) and PINK1 knockout mice received stereotaxic injection of AA-viruses expressing GFP (n=7), hPINK1 (n=5) or hParkin (n=13) in their striatum 2 weeks before being sacrificed. **(C)** Representative picture of the brain slices expressing GFP-hParkin, shown using GFP fluorescence. **(D)** Quantification of the infarct volume in the brain slices (* $p<0.05$ ** $p<0.01$ and *** $p<0.001$).

(A) Wild type

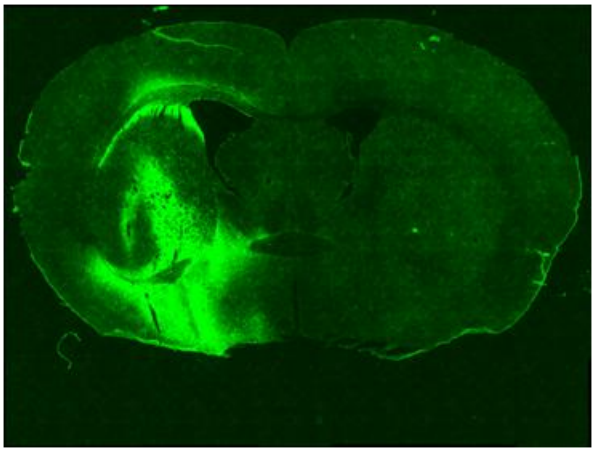
GFP **hPINK1** **hParkin**



(B) PINK1 knockout



(C)



(D)

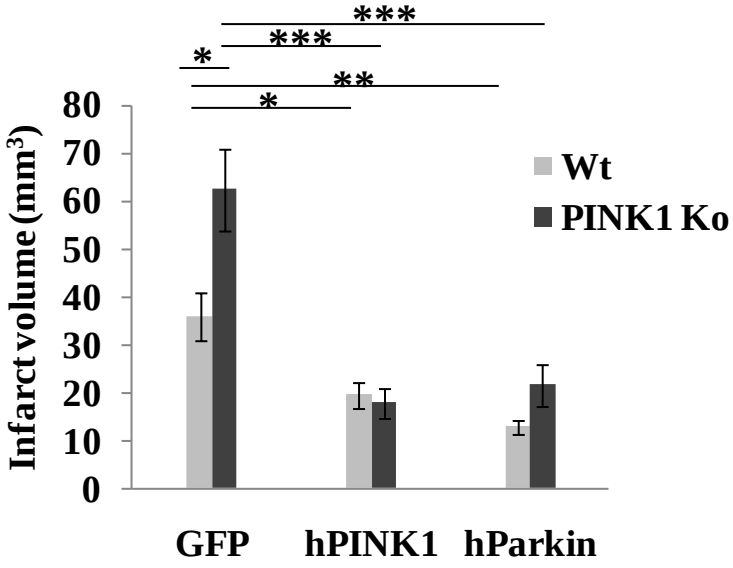


Figure 3.11

3.2. Mechanism of action

The current model in which PINK1 functionally interacts with Parkin to exert quality control of mitochondria is well accepted in many laboratories. In this model, turnover of damaged mitochondria secures mitochondrial homeostasis, and promotes neuronal survival. Understanding how this pathway may be involved in the pro-survival functions of these two PD genes is very important since it could provide better understanding of the injury process. In this study, we examined this view through challenging cortical neurons by different stressors that mimic the ischemic condition. However, my data indicates that this pathway is likely dispensable for the neuroprotective role of PINK1 and Parkin in ischemic context.

3.2.1. Parkin is not translocated to damaged mitochondria in response to glutamate treatment as a model of ischemia.

Since oxidative stress and mitochondrial dysfunction have been implicated as driving forces for Parkin mitochondrial translocation, we examined if hypoxia or glutamate treatment could induce Parkin translocation to damaged mitochondria. To do so, we infected cultured cortical neurons with adeno-associated viral vectors expressing GFP-fused hParkin or control GFP at the time of plating. As previously described, media from the wells were replaced with fresh growth media without supplementary antioxidants on DIV9 (Joselin et al., 2012). The day after, cortical neurons were subjected to glutamate treatment. We have also employed CCCP treatment as a positive control. Consistent with our previous studies, CCCP treatment could induce a substantial Parkin translocation to the

mitochondria (Joselin et al., 2012) **(Fig. 3.12A, 3.12C)**. Co-localization of hParkin and mitochondria was confirmed by immunofluorescence studies using confocal microscopy. As shown in **Figure 3. 12.,** in spite of mitochondrial fragmentation induced by glutamate treatment or hypoxia, cortical neurons did not display any Parkin translocation to the mitochondria. We examined various time points for glutamate treatment including 0.5 , 1 and 2 hrs glutamate treatment followed by 0 , 0.5 , 1 , 2 , 3 or 4 hrs incubation period in conditioned medium for all time points. Here, we have presented the results for the experiment in which 1 hr glutamate treatment followed by 1 hr incubation period was employed **(Fig. 3.12B)**. We chose this representative time point because it was the effective time point for the survival assay in my study **(Fig. 3.3)**.

We also employed hypoxia to produce both excitotoxic and delayed (apoptotic-like) cell death. For excitotoxic model of death, we employed 1 , 2 , 3 , and 4 hrs hypoxia in the absence of MK801, followed by 0 , 1 , 2 , 3 , and 4 hrs re-oxygenation for all time points. For delayed model of death, neuronal cultures were subjected to 12, 18, and 24 hrs hypoxia in the presence of MK801 followed by 0, 12, 18 or 24 hrs re-oxygenation for all time points. We have presented the results of the experiment where neurons received 16 hrs hypoxia followed by 12 hrs re-oxygenation **(Fig. 3.12A).** This time point was sufficient to induce about 40% neuronal death. Then cultured cortical neurons were extensively assessed for Parkin mitochondrial translocation by confocal microscopy. We have also examined cultured CGNs in the same experimental setting. In the wide ranges of experimental time points, we did not find any evidence of formation of Parkin puncta as an indicator of Parkin mitochondrial translocation.

Figure 3.12. Parkin translocation to the mitochondria does not occur in cortical neurons treated with glutamate or hypoxia. (A&B) Representative confocal images of cortical neurons infected with AAV-viruses carrying GFP-hParkin at the time of plating; treated with 5 µM CCCP for 2 hrs or 200µM glutamate for 2 hrs in the absence or presence of MK801, or 16 hrs hypoxia in the presence of MK801, followed by 12 hrs re-oxygenation. In all conditions, culture media was replaced with fresh media without antioxidant supplement B27, 24 hrs before indicated treatments. **(C)** Quantification of number of neurons displaying GFP–hParkin translocation to mitochondria. Results are representative of three independent experiments.

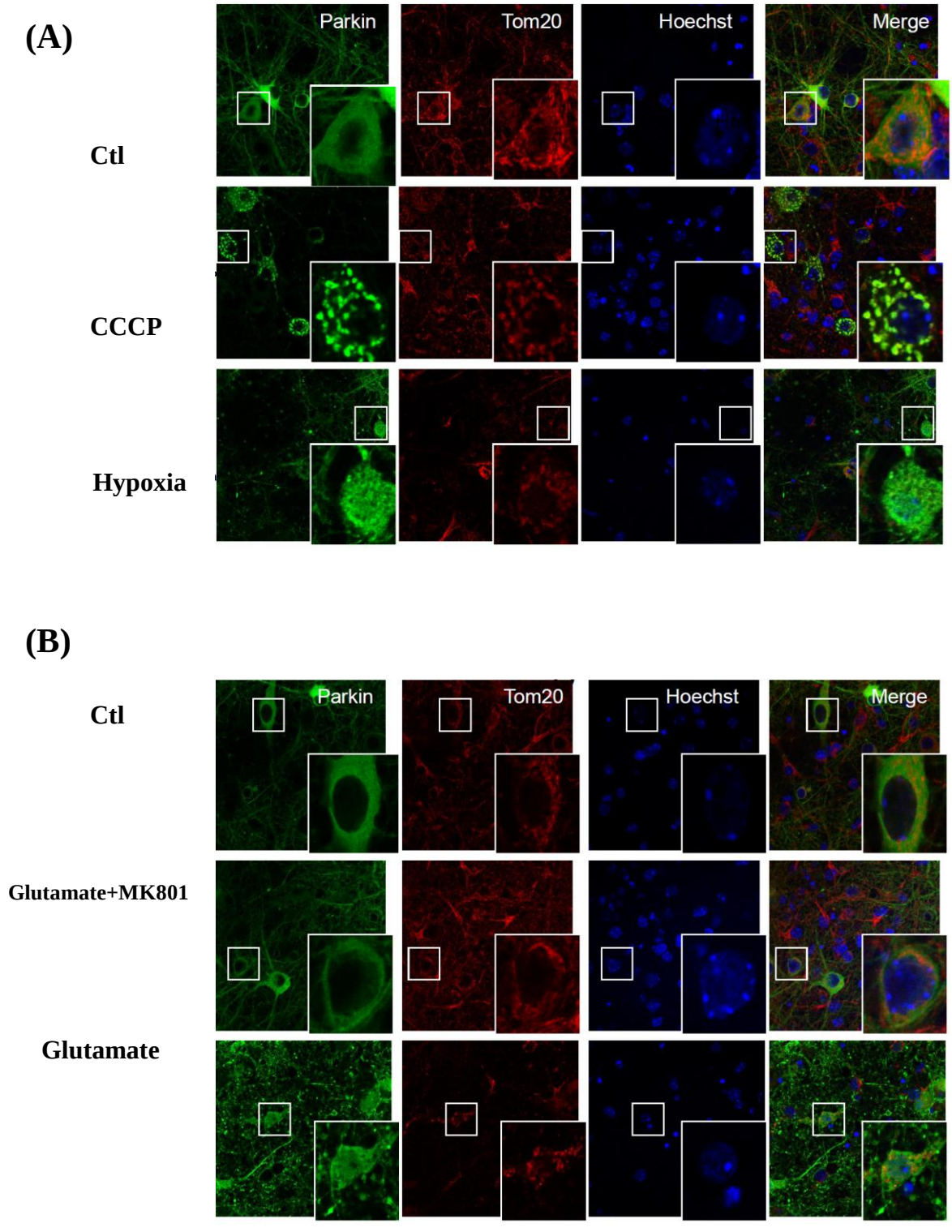

(A)
Parkin
Tom20
Hoechst
Merge
Ctl
CCCP
Hypoxia
(B)
Parkin
Tom20
Hoechst
Merge
Ctl
Glutamate+MK801
Glutamate

Figure 3.12

(C)

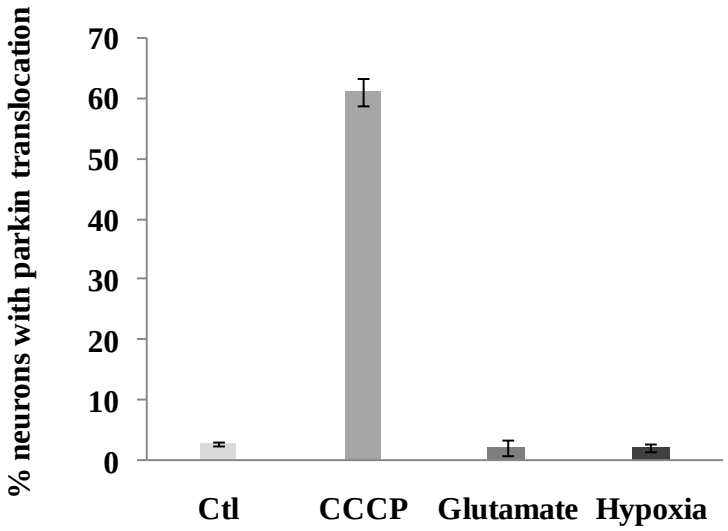


Figure 3.12 (continue)

3.2.2. Phosphorylation of AKT in response to excitotoxicity is reduced in the absence of PINK1 and Parkin.

As demonstrated above, we could not find any evidence indicating that Parkin mediates PINK1 neuroprotective effect through translocation of Parkin to mitochondria and the mitochondrial quality control pathway. However, our results also indicate that cytosolic PINK1 exerts neuroprotective effect after glutamate treatment.

In light of the above findings, together with the knowledge that AKT is part of the survival pathway that is activated after ischemic insult, we examined the hypothesis that PINK1 might act as an upstream regulator of AKT pathway to protect against ischemic injury. To test this hypothesis, I first determined whether lack of PINK1 affects AKT phosphorylation following glutamate treatment. Similarly, I also examined if Parkin deficiency affects the level of phosphorylation of AKT. To examine this, neurons harvested from PINK1 or Parkin knockout and WT embryos were treated with 200 μM glutamate for the indicated times periods.

Interestingly, in our experimental paradigm of neuronal death induced by glutamate treatment, phosphorylation of AKT (pAKT) was up-regulated. This up-regulation was diminished in the absence of PINK1 or Parkin **(Fig. 3.13)**. Conversely, we also found that over-expression of human PINK1 or Parkin (hPINK1, hParkin) enhances the level of phosphorylation of AKT in response to glutamate treatment **(Fig. 3.14)**.

To further investigate if PINK1 and Parkin function in the same pathway, we over-expressed hPINK1 in Parkin deficient cortical neurons. Interestingly, in response to excitotoxicity, hPINK1 partially rescued the pAKT loss observed with Parkin deficiency. **(Fig. 3.15)**. However, over-expression of hParkin could not induce the same effect in PINK1 deficient neurons **(Fig. 3.16)**. Expression of hPINK1 or hParkin was confirmed in all experiments required PINK1 or Parkin expression. However, it is important to mention that there are no commercial antibodies that can detect either endogenous mouse PINK1 or Parkin.

Figure 3. 13. Phosphorylation of AKT is reduced in PINK1 or Parkin deficient cortical neurons in response to cytotoxicity. Cortical neurons of 14-16 days old **(A)** PINK1 or **(B)** Parkin knock out, and WT embryos (10DIV) were treated with 200 µM glutamate in a time-dependant fashion. Neuronal extracts were subjected to western blot analysis and probed for pAKT (S473), total AKT and β-actin. Quantification of pAKT protein level based on its ratio to total AKT, corrected with β-actin for loading in PINK1 **(C)** and Parkin **(D),** WT and Ko neuronal extracts. Quantification of pAKT protein level corrected with β-actin for loading in PINK1 **(E)** and Parkin **(F),** WT and Ko neuronal extracts (n=3).

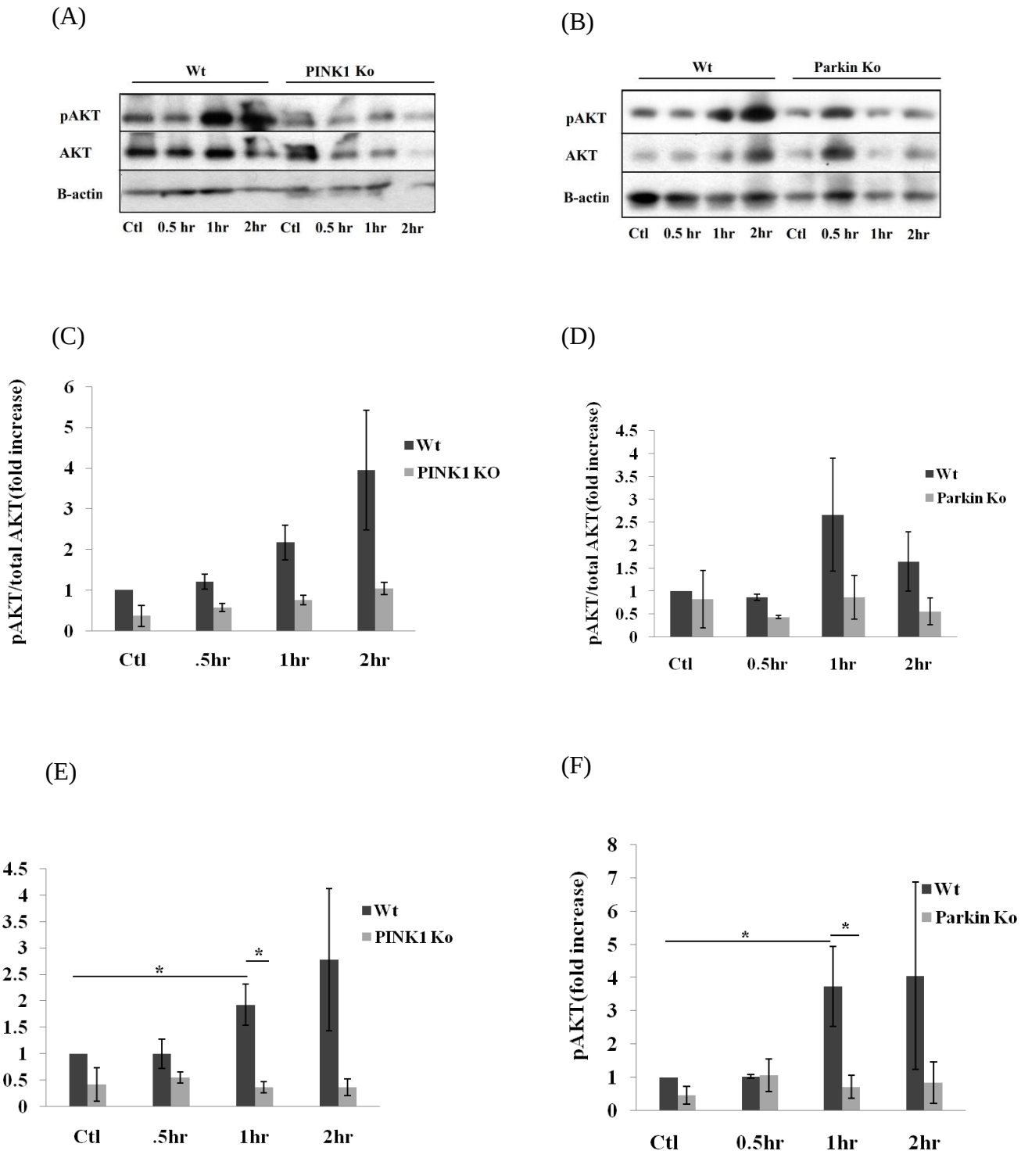
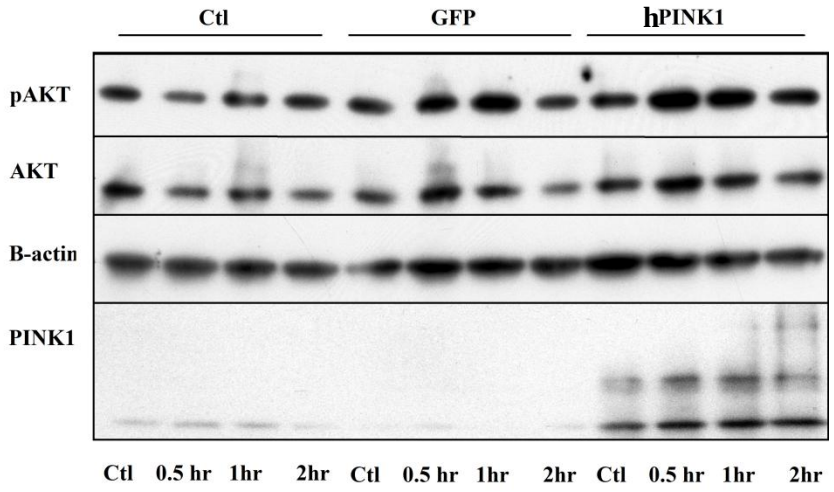

(A)
Wt
PINK1 Ko
pAKT
AKT
B-actin
Ctl 0.5 hr 1hr 2hr Ctl 0.5 hr 1hr 2hr
(B)
Wt
Parkin Ko
pAKT
AKT
B-actin
Ctl 0.5 hr 1hr 2hr Ctl 0.5 hr 1hr 2hr
(C)
pAKT/total AKT(fold increase)
Wt
PINK1 KO
Ctl .5hr 1hr 2hr
(D)
pAKT/total AKT(fold increase)
Wt
Parkin Ko
Ctl 0.5hr 1hr 2hr
(E)
pAKT(fold increase)
Wt
PINK1 Ko
Ctl .5hr 1hr 2hr
(F)
pAKT(fold increase)
Wt
Parkin Ko
Ctl 0.5hr 1hr 2hr

Figure 3.13

Figure 3.14. Over-expression of hPINK1 or hParkin enhances phosphorylation of AKT in cortical neurons in response to cytotoxicity. Over-expression of either **(A)** GFP-hPINK1 or **(B)** GFP- hParkin, along with GFP as control, in cortical neurons of 14-16 days old WT embryos at time of plating, followed by glutamate treatment (10DIV, 200 µM) for the indicated time course. Neuronal extracts were subjected to western blot analysis and probed for pAKT (S473), total AKT, β-actin, hPINK1 and hParkin. Quantification of pAKT protein level based on its ratio to total AKT (just for 1 hr time point), corrected with β-actin for loading in neuronal extracts expressing hPINK1 **(C)** and hParkin **(D)** compared with that in non-infected control or GFP expressing samples. Quantification of pAKT protein level (just for 1 hr time point), corrected with β-actin for loading in neuronal extracts expressing hPINK1 **(E)** and hParkin **(F)** compared with that in non-infected control or GFP expressing samples (n=3).

(A)



(B)

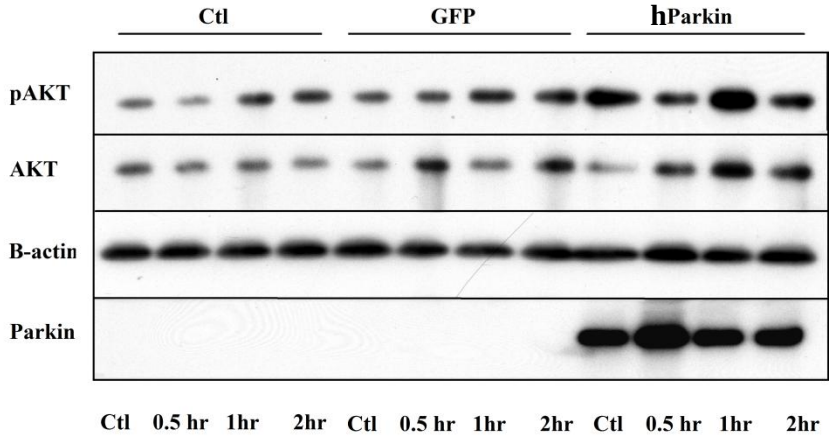
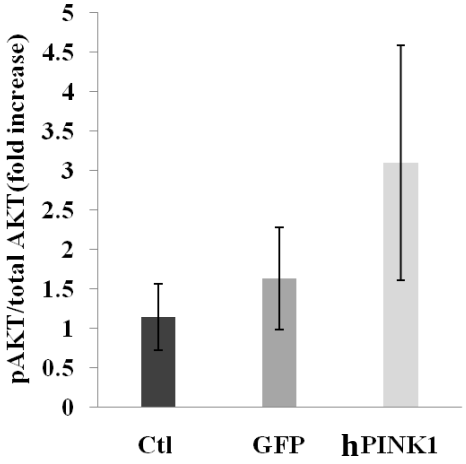
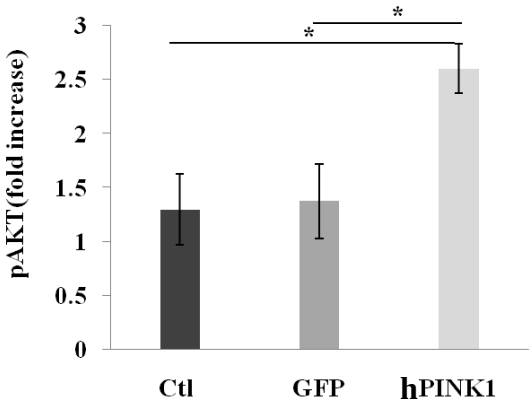


Figure 3.14

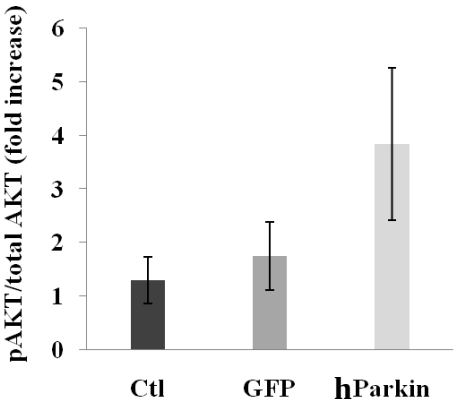
(C)



(E)



(D)



(F)

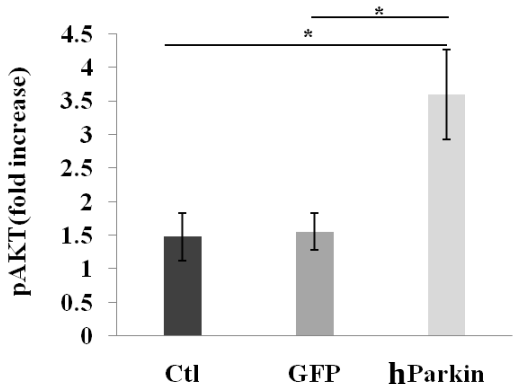
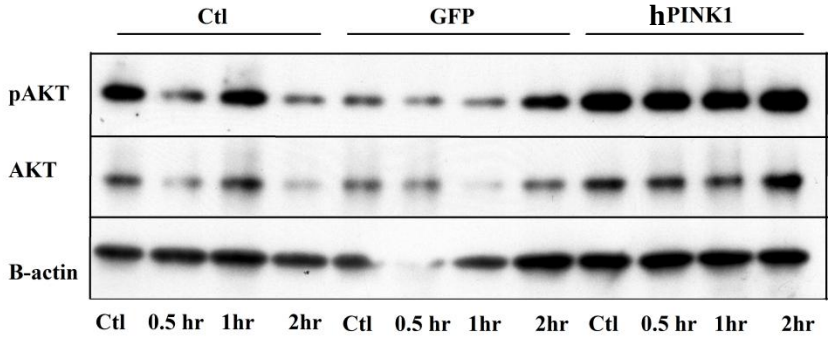


Figure 3.14 (continue)

Figure 3.15. Over-expression of hPINK1 or hParkin enhances phosphorylation of AKT in Parkin deficient cortical neurons in response to cytotoxicity. Over-expression of either **(A)** GFP-hPINK1 or **(B)** GFP- hParkin, along with GFP as control in cortical neurons of 14-16 days old Parkin deficient embryos at the time of plating, followed by glutamate treatment (10DIV, 200 μM) for the indicated time course. Neuronal extracts were subjected to western blot analysis and probed for pAKT (S473), total AKT and β-actin. Quantification of pAKT protein level based on its ratio to total AKT (just for 1 hr time point), corrected with β-actin for loading in neuronal extracts expressing hPINK1 **(C)** and hParkin **(D)** compared with that in non-infected control or GFP expressing samples. Quantification of pAKT protein level (just for 1 hr time point), corrected with β-actin for loading in neuronal extracts expressing hPINK1 **(E)** and hParkin **(F)** compared with that in non-infected control or GFP expressing samples (n=3).

(A)



(B)

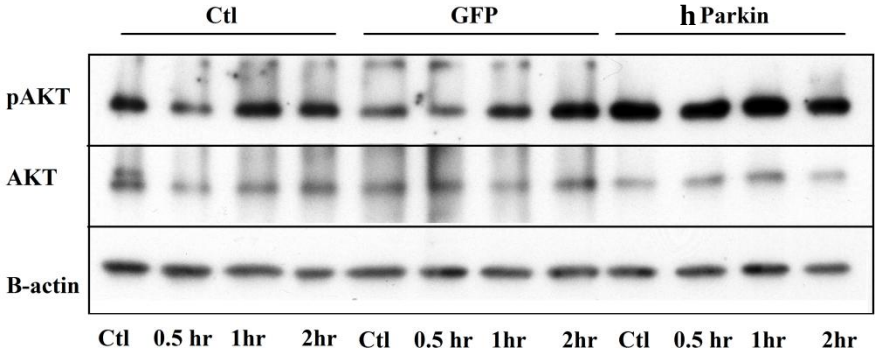
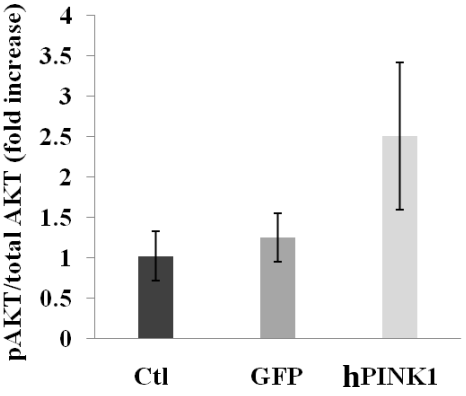
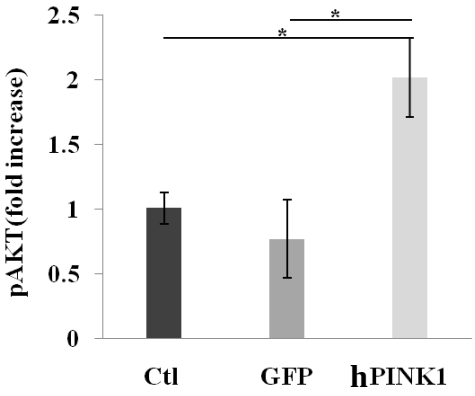


Figure 3.15

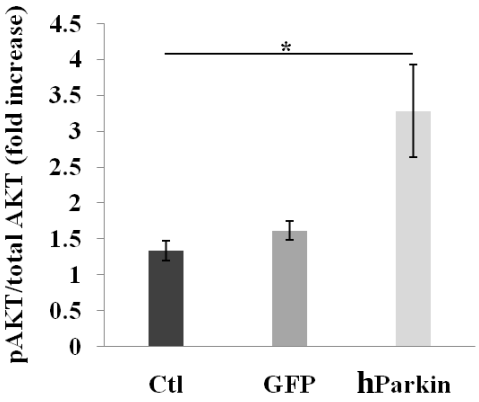
(C)



(E)



(D)



(F)

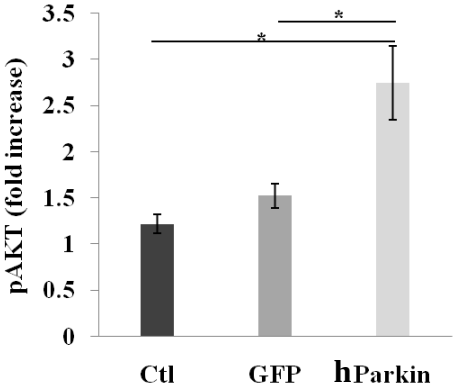
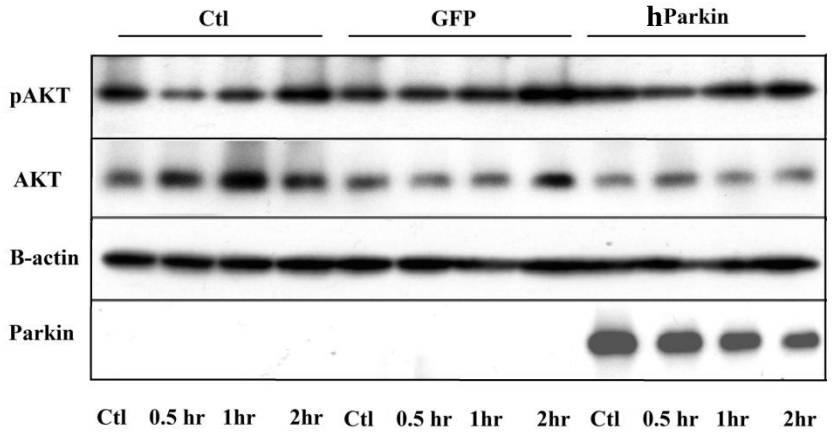


Figure 3.15 (continue)

Figure 3.16. Over-expression of hParkin does not enhance phosphorylation of AKT in PINK1 deficient cortical neurons in response to cytotoxicity. (A) Over-expression of either GFP- hParkin or GFP as control, in cortical neurons of 14-16 days old PINK1 deficient embryos at time of plating, followed by glutamate treatment (10DIV, 200 µM) for the indicated time course. Neuronal extracts were subjected to western blot analysis and probed for pAKT (S473), total AKT, β-actin and hParkin.**(B)** Quantification of pAKT protein level based on its ratio to total AKT (just for 1 hr time point), corrected with β-actin for loading in neuronal extracts expressing hParkin compared with that in non-infected control or GFP expressing samples (n=3).

(A)



(B)

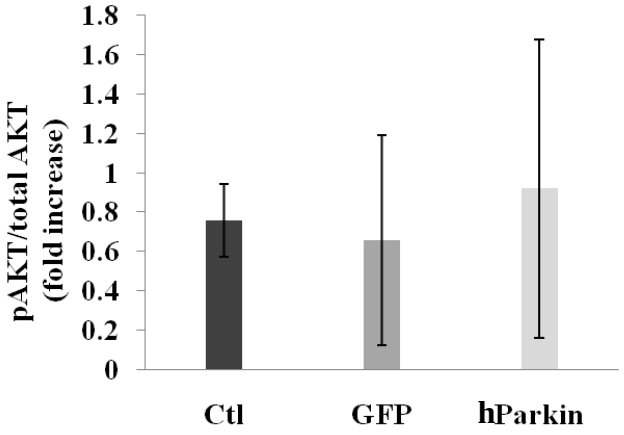


Figure 3.16

3.2.3. PINK1 regulates AKT phosphorylation through mTOR signaling pathway to exert its pro-survival function in ischemia context.

Evidence has been previously reported indicating that PINK1 enhances the level of pAKT through the mTOR pathway (Murata et al., 2011). To assess if that was the case in our experimental paradigms, we employed an mTOR inhibitor (Rapamycin) to test if PINK1 or Parkin targets the mTOR pathway to introduce their neuroprotective function through the AKT pathway. Briefly, eight days after plating, we pre-treated cortical neurons with 1 μM Rapamycin or its vehicle for 48 hrs before glutamate treatment. After glutamate treatment, the neurons were assessed for survival. Additionally, a conventional pharmacological inhibitor of AKT, LY294002 (10 μM) was used for one hr before glutamate treatment as a positive control (Aleyasin et al., 2010). Our results confirmed that both LY294002 (10 μM, 1 hr) and Rapamycin (1 μM, 48 hrs) could efficiently inhibit phosphorylation of AKT in cortical neurons exposed to 1 hr glutamate treatment **(Fig. 3.17)**. Importantly, Parkin over-expression did not overcome the inhibitory effect of LY294002 (10 μM, 2 hrs) or Rapamycin (1 μM, 48 hrs) over the phosphorylation level of AKT **(Fig. 3.17)**.

My results demonstrate that treatment with Rapamycin significantly reduces the neuroprotective effect of PINK1, suggesting that PINK1 acts, at least partially, through the mTOR pathway to promote neuronal survival after glutamate treatment **(Fig. 3.18)**. As expected, the survival rate was significantly reduced in PINK1 deficient neurons**(Fig. 3.19)**. It is noteworthy that Rapamycin treatment did not induce further reduction in survival rate

of PINK1 deficient neurons compared with that of non-treated PINK1 deficient neurons, further suggesting that PINK1 functions through the mTOR pathway. Interestingly, over-expression of hParkin could not rescue the sensitivity of PINK1 deficient neurons against Rapamycin treatment **(Fig. 3.19)** This finding is in line with our result showing that over-expression of Parkin could not enhance AKT phosphorylation in PINK1 deficient neurons.

Figure 3.17. Over-expression of hParkin could not reduce the inhibitory effect of LY249002 or Rapamycin on the level of phosphorylation of AKT in cortical neurons. Over-expression of either GFP- hParkin or GFP as control, in cortical neurons of 14-16 days old WT embryos at time of plating, treated by either LY249002 (10DIV, 1 hr, 10 µM) or Rapamycin (8DIV, 48 hr, 1µM). The treated neurons were subjected to 1 hr glutamate treatment. Neuronal extracts were subjected to western blot analysis and probed for pAKT (S473), total AKT, β-actin, and hParkin.

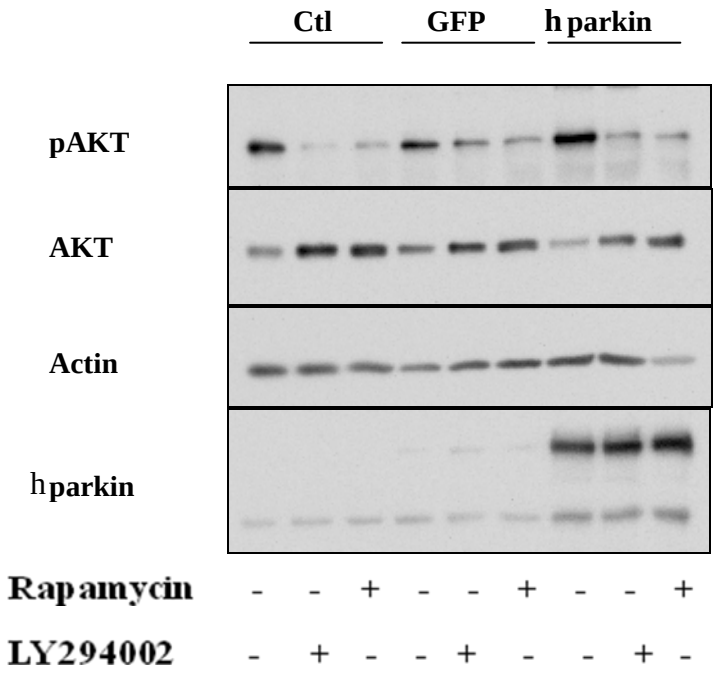


Figure 3.17

Figure 3.18. Rapamycin reduces the neuroprotective effect of PINK1 against glutamate treatment *in vitro*. Cortical neurons were harvested from 14-16 days old embryos. Eight days after plating, cortical neurons were infected with rAV expressing either GFP or GFP-hPINK1, and the day after, neurons were treated with Rapamycin (1µM, 48 hrs). After two days, neurons were transiently treated with 200 µM glutamate in the presence and absence of 15 µM MK801 for 60 minutes, followed by 60 minutes incubation period. Glutamate was then washed out from the treated wells, and neurons were fixed with Lana's buffer. Plates were stored and examined in the cold room for survival assessment. Non-treated neurons were used as the control group to determine the survival rate. (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$)

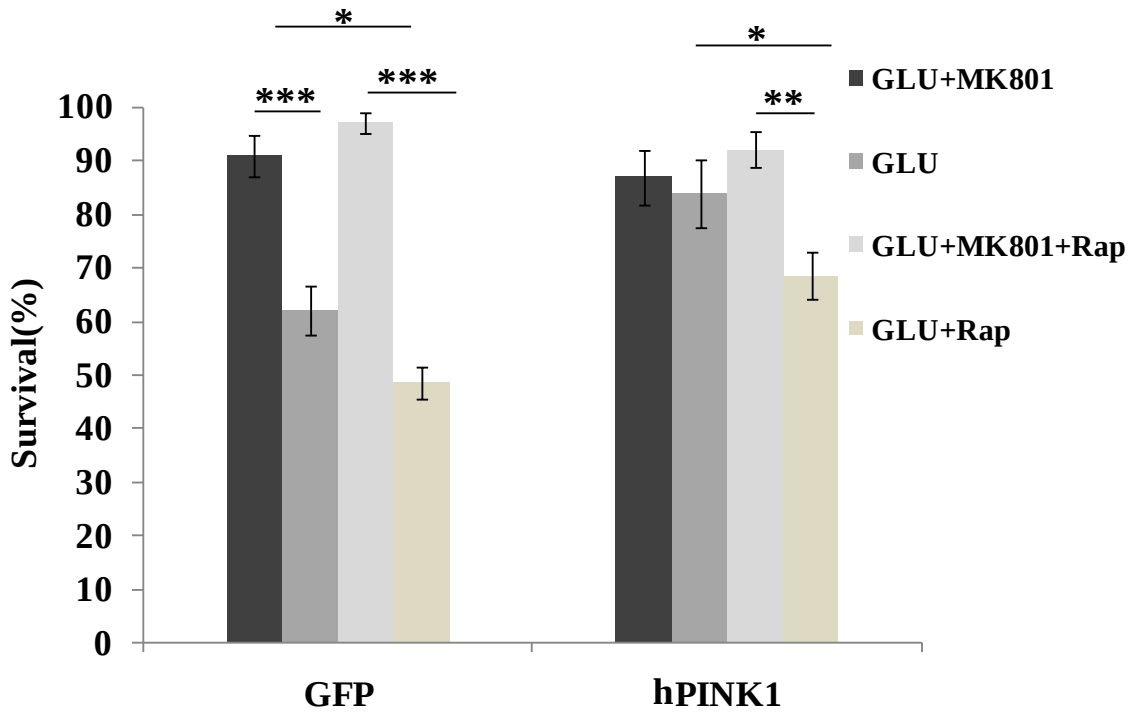

*
*
***
***
**
GLU+MK801
GLU
GLU+MK801+Rap
GLU+Rap
Survival(%)
100
90
80
70
60
50
40
30
20
10
0
GFP
hPINK1

Figure 3.18

Figure 3. 19. Rapamycin reduces the neuroprotective effect of Parkin against glutamate treatment but it does not cause further reduction in survival rate in PINK1 deficient neurons. Cortical neurons harvested from 14-16 days old WT and PINK1-null embryos were infected with either rAAV expressing GFP or GFP-hParkin. Neurons (8DIV) were then treated with Rapamycin (1µM, 48 hrs). After two days, neurons were transiently treated with 200 µM glutamate in the presence and absence of 15 µM MK801 for 60 minutes, followed by 60 minutes incubation period. Finally, glutamate was washed out from the treated wells, and neurons were fixed with Lana's buffer. Non-treated neurons were used as the control group to determine the survival rate. (* $p<0.05$)

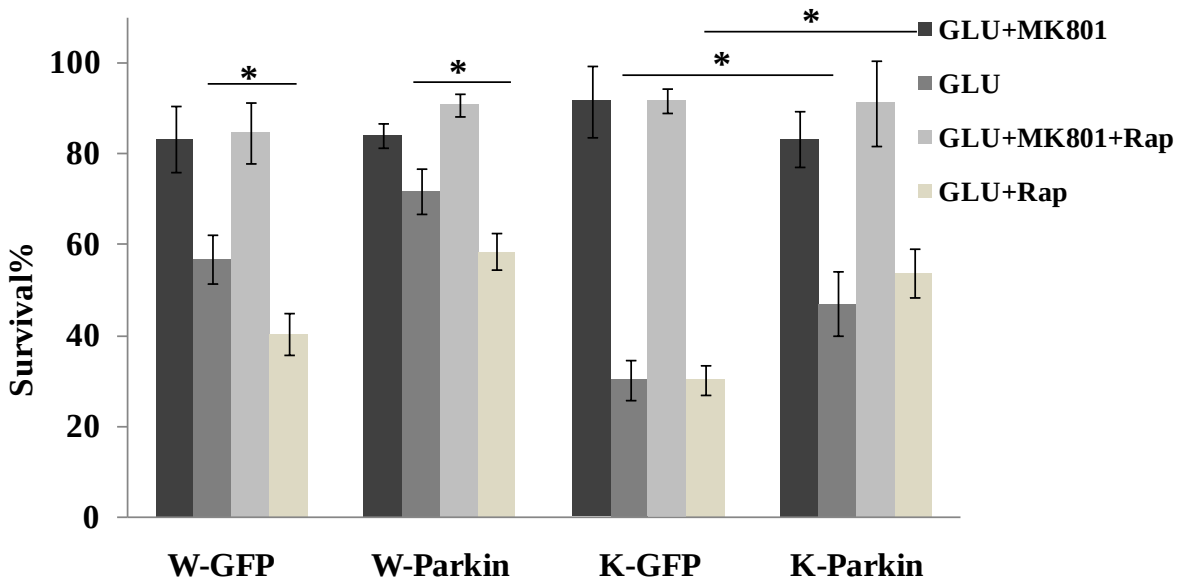

Survival%
100
80
60
40
20
0
*
*
*
*
GLU+MK801
GLU
GLU+MK801+Rap
GLU+Rap
W-GFP
W-Parkin
K-GFP
K-Parkin

Figure 3.19

Chapter 4

General discussion

4. Discussion:

4.1. Summary

Over the last few decades, remarkable progress has been made in our knowledge of the pathogenesis of ischemic brain injury. However, due to the complexity and dynamic nature of its pathophysiology, our understandings have largely failed to translate into effective therapeutic outcomes. For instance, medical interventions targeting either the acute or subacute aspects of the ischemic cascade, such as glutamate release and neuroinflammation, have not produced any significant improvement in the outcomes in clinical trials (Ceulemans et al., 2010; Lai et al., 2014; Tobin et al., 2014). One possible explanation for these trial failures is the notion that these neuronal responses have dual functions, which makes it difficult to modulate them in favour of neurosurvival. In fact, these elements appear to play both detrimental and neuroprotective roles, depending on the factors that get involved or the temporal pattern of their activation. Therefore, better understanding of the molecular pathways underlying these dual functions is necessary to target them more specifically.

In this regard, a number of novel putative molecular pathways have been studied in our lab in the hope to identify new therapeutic strategies for ischemic injury. For instance, we have previously shown that Parkinson's disease gene DJ-1 plays a neuroprotective role against ischemia through its anti-oxidant properties (Aleyasin et al., 2007). Additionally, in an animal model of PD, we have shown that DJ-1 protects dopaminergic neurons from the neurotoxin MPTP by modulation of the AKT pathway; a major neuronal pro-survival

pathway induced upon ischemia (Aleyasin et al., 2010). Moreover, in a series of innovative stroke studies, we have identified cell cycle signals as critical mediators of ischemic neuronal death (Iyirhiaro et al., 2008; Iyirhiaro et al., 2014; Rashidian et al., 2005; Rashidian et al., 2009).

The work presented in this thesis also identifies two other Parkinson's disease genes, PINK1 and Parkin, as critical mediators of neuronal survival pathways, which are activated by ischemia. In the hope of elucidating the mechanism mediating PINK1/Parkin neuroprotective function, we explored the role of PINK1/Parkin mitochondrial quality control (MQC) pathway in neuronal response to stroke insult, however, our results rule out the involvement of this pathway. On the other hand, we found some evidence indicating that AKT pro-survival pathway is, at least partially, involved in the neuroprotective function of PINK1/Parkin in ischemia.

In the following section, we have briefly summarized and interpreted the major findings presented in the thesis. We have also discussed their implications and potential contributions to the field.

4.2. Overview of major findings

Parkinson's disease is primarily considered to be sporadic in nature, a concept that has been substantially changed by emerging evidence linking a genetic component to the disease. While familial Parkinsonism affects a small minority of cases, comprehensive study of the genetic factors has enormously contributed to our understanding of the disease.

Particularly, we have gathered invaluable knowledge about their roles in the cells from teasing out PD-genes in a context different from the one in which they were originally identified. Out of these, Parkinson's disease gene PINK1 is the target of interest in this study.

Therefore, the principal topic of the present study was to address important questions about the physiological and molecular functions of PINK1 in stroke context, namely: does PINK1 have any role in pro-survival mechanism against ischemia? What is the significance of the PINK1/Parkin MQC pathway in stroke context? What is the significance of cytosolic PINK1 in neuronal pro-survival response to ischemia, and finally which mechanism is driven by PINK1 in the cytosol?

Early studies have described a protective role for PINK1 in multiple cell types and animal experimental models (Chen et al., 2015; Dagda et al., 2009; Haque et al., 2012; Haque et al., 2008; Zhou et al., 2014). In this thesis, we identified a clear neuroprotective role for PINK1 against ischemia/reperfusion injury. In addition, in line with previous studies from our lab as well as others, the results presented in chapter three indicate that in the ischemia context, PINK1 likely mediates its pro-survival effects through its kinase activity (Haque et al., 2008; Petit et al., 2005; Sim et al., 2006). How PINK1 mediates this protective effect is unclear.

Several lines of evidence have suggested that the role of PINK1/Parkin axis in mitochondrial quality control may be responsible for their neuroprotective function (Jin and Youle, 2013; Koyano et al., 2014). Considering that PINK1 mitochondrial localization is

important for proper function of the PINK1/Parkin MQC pathway, we investigated the importance of mitochondrial localization in PINK1 neuroprotective function. Interestingly, our results indicate that cytosolic PINK1 [PINK1 with deleted mitochondrial motif (ΔN)] resumes its pro-survival function therefore; mitochondrial localization is not necessary for PINK1 pro-survival function in ischemic injury context. This data was the first piece of evidence indicating that the PINK1/Parkin MQC pathway might be dispensable from the neuroprotective effect of PINK1 in our experimental model. However, the current knowledge in the field indicates that in response to mitochondrial membrane depolarization, full-length PINK1 is translocated to the mitochondrial outer membrane with its kinase domain facing the cytosol to phosphorylate its cytosolic targets (Narendra et al., 2010; Silvestri et al., 2005). Additionally, it has been reported that upon mitochondrial depolarization, PINK1 mitochondrial import is blocked, resulting in accumulation of full length PINK1 in the outer mitochondrial membrane (Meissner et al., 2011). These findings suggest the possibility that PINK1 kinase domain exert its neuroprotective function while either residing at the mitochondrial outer membrane or in some cases, free-floating in the cytosol in the vicinity of its mitochondrial or cytosolic targets. Therefore, considering these possibilities, we did not completely rule out the role of the PINK1/Parkin MQC pathway in the neuroprotective effect of PINK1, at this stage.

Therefore, we sought to further examine the relevance of the PINK1/Parkin MQC to mitochondrial well being and neuronal survival by challenging neurons in ischemia context. This aim was particularly important given that the physiological significance of MQC

pathway, and particularly its relevance to pathogenesis of neurological disease such as PD and stroke, is not clear.

Generally, translocation of Parkin to defective mitochondria, and activation of MQC pathway can be induced by chemicals or uncoupler proteins such as CCCP and UCP peptides. In the context of immortalized cell lines, mitochondrial depolarization is sufficient to induce Parkin translocation to the damaged mitochondria while in neuronal cell culture, it fails to induce significant mitochondrial Parkin translocation in the presence of antioxidant supplements (Grenier et al., 2013; Joselin et al., 2012). Interestingly, in the absence of antioxidant supplements in neuronal culture media, a variety of oxidative agents such as H_2O_2, rotenone and MPP^+ can induce recruitment of Parkin to depolarized mitochondria (Joselin et al., 2012). These compounds appear to induce mitochondrial membrane depolarization, accumulation of misfolded proteins and oxidative stress (Joselin et al., 2012; Narendra et al., 2010). Similar adverse events have been shown to be induced after ischemic insult, and theoretically are expected to induce Parkin mitochondrial translocation.

Accordingly, in a number of experimental paradigms involving both excitotoxic and more delayed form of neuronal insult, we examined if Parkin regulated MQC is involved in neuroprotective function of PINK1 in ischemic context. Although our results demonstrate that PINK1 and Parkin functionally interact to protect neurons against ischemia, we did not detect any Parkin puncta formation in response to glutamate treatment or hypoxia. This suggests that Parkin recruitment to damaged mitochondria may not be involved in pro-

survival function of PINK1 in our experimental paradigm. Additionally, despite the important role of PINK1 in the PINK1/Parkin MQC pathway, our data also indicates that Parkin could rescue the sensitivity of PINK1 deficient neurons to excitotoxicity. In this regard, previous evidence has shown that even in the presence of exogenous Parkin expression, PINK1 is required for Parkin translocation (Joselin et al., 2012; Narendra et al., 2010). Accordingly, our data that Parkin can rescue in the absence of PINK1 is inconsistent with a model by which a PINK1/Parkin regulated MQC pathway plays a significant role in ischemic death. Considering that mitochondrial fragmentation, oxidative stress and mitochondrial depolarization are presumably prominent elements induced by glutamate treatment and hypoxia (Martin et al., 1994), it was surprising not to detect any Parkin mitochondrial translocation following glutamate treatment and hypoxia in the present study.

Thus, a major question remains unanswered: What is the main driving force for Parkin recruitment to the damaged mitochondria in neurons? It appears that unexplored variables are at play to orchestrate this phenomenon. Therefore, further studies, including semi quantitative measurements of ROS level or mitochondrial charge, could give us a base to grade and compare these factors in different models of neuronal injury. These systematic measurements would potentially help to figure out if this phenomenon merely occurs upon complete mitochondrial depolarization and not disease- relevant conditions, or whether an optimal combination of these factors is needed for activation of PINK1/Parkin MQC pathway and induction of Parkin recruitment to mitochondria.

Just as an example to clarify this view, it has been shown that induction of mitochondrial uncoupling proteins (UCPs) can protect neurons from oxidative stress by blocking ROS generation (Horvath et al., 2003a). Uncoupling proteins are inner mitochondrial membrane proteins that naturally uncouple oxidative phosphorylation and ATP synthesis to regulate mitochondrial energy metabolism and ROS generation (Horvath et al., 2003b). Interestingly, it has been shown that induction of UCP2 protects neurons against ischemia and traumatic brain injury through activation of neuronal redox signaling or suppression of caspase activity (Mattiasson et al., 2003). Both ischemia and traumatic brain injury are involved in brain oxidative damage. Based on these findings, one can speculate that in the course of ischemia, mitochondrial depolarization induced by activation of UCPs can activate redox signaling. Theoretically, this neuroprotective mechanism can bypasse the PINK1/Parkin MQC pathway, or even suppresse this pathway to protect neurons through a pathway distinct of PINK1/Parkin MQC pathway and mitophagy. Therefore, it appears that physiological uncouplers such as UCPs activate different responses than that activated by synthetic uncouplers such as CCCP and FCCP. This notion highlights the importance of systematic evaluations that we proposed above. However, it is important to note that, generally, it is hard to interpret negative data and we cannot absolutely rule out PINK1/Parkin MQC involvement in stroke.

Nevertheless, with regard to mitochondrial homeostasis, pro-survival function of PINK1 could be mediated through alternative mitochondrial pathways. For instance, PINK1 has been detected in different cellular (e.g. cytosol, mitochondria) and subcellular (e.g. mitochondrial outer membrane, inner membrane and intermembrane space)

compartments pointing to its multiple functional attributes. Interesting work from our lab has identified an important role for PINK1 in regulation of mitochondrial calcium homeostasis in connection with a mitochondrial calcium antiporter Letm1 (Huang et al., unpublished data). Since, Letm1 resides in inner mitochondrial membrane, PINK1 most likely needs to be stabilized in inner mitochondrial membrane (or its vicinity) to interact with this protein. Therefore, this pathway most likely introduces a distinct mechanism than that of PINK1/Parkin dependent MQC, in which PINK1 is primarily needed to be translocated to the mitochondrial outer membrane.

Considering the critical role of calcium imbalance in ischemia induced neuronal injury, and important role of PINK1 in regulating mitochondrial calcium homeostasis, it would be particularly interesting to investigate neuroprotective role of PINK1 by working through this pathway in ischemic context as an alternative mitochondrial neuroprotective pathway.

As mentioned above, consistent with previous studies from our lab, we have found that cytosolic PINK1 is neuroprotective against ischemic insult, suggesting a putative cytosolic pathway for PINK1 signalling (Haque et al., 2008). In support of this, a recent report indicated that PINK1 phosphorylates the Rictor subunit of mTORC2 complex to enhance AKT phosphorylation (Murata et al., 2011). Additionally, it has been reported that Parkin may also act upstream of PI3K pathway to permit AKT phosphorylation (Fallon et al., 2006). Therefore, we evaluated a potential mechanism through which PINK1 and Parkin might activate AKT through a cytosolic pathway. In order to confirm this

hypothesis, we addressed these two important questions: (i) whether AKT activity (the level of pAKT) was changed in the absence of PINK1 or Parkin, particularly in ischemic context (ii) whether AKT functionally participated in the pro-survival pathways activated by PINK1 or Parkin.

Accordingly, in our excitotoxic model of neuronal death induced by glutamate treatment, we found that phosphorylation of AKT (pAKT) was up-regulated, and this increase was partially dependent on PINK1 and Parkin. Interestingly, over-expression of PINK1 could rescue the adverse effect of Parkin deficiency on the level of phosphorylation of AKT but the reverse was not true, suggesting that Parkin requires PINK1 to induce AKT phosphorylation. These findings indicate that the neuroprotective function of Parkin through AKT pathway is potentially PINK1 dependent. Our results also demonstrated that Rapamycin significantly reduces the neuroprotective effect of PINK1, suggesting that PINK1 acts through the mTOR pathway. However, administration of Rapamycin could not completely abolish the neuroprotective effect of PINK1, suggesting that PINK1 may exert its pro-survival function through other pathway(s) in addition to the mTOR pathway. Consistent with the above findings, Rapamycin did not diminish the pro-survival effect induced by over-expression of Parkin in PINK1 deficient neurons, suggesting that Parkin rescues PINK1 deficiency through a mechanism distinct of the mTOR/ AKT pathway. One possible explanation is that Parkin and PINK1 act through two different converging pathways to regulate the prosurvival function of AKT. As illustrated in **Figure 4.1**, AKT contains several phosphorylation sites including two phosphorylation sites on T308 and S473 residues. Phosphorylation of AKT on these two residues has been suggested to be

important for its activity (Alessi et al., 1997; Zhao et al., 2006b). T308 is phosphorylated through the PI3K/ PDK1 pathway, whereas S473 is phosphorylated by mTORC2 (Costa-Mattioli and Monteggia, 2013; Zhao et al., 2006b). Phosphorylation of T308 residue has been shown to be necessary and sufficient for AKT activity (Alessi et al., 1997; Vivanco and Sawyers, 2002). However, phosphorylation of S473 residue has been shown to precede and enhance AKT phosphorylation on T308 residue (Sarbassov et al., 2005). Therefore consistent with previous studies, we suggest a model by which Parkin acts through the PI3K pathway to regulate AKT phosphorylation on T308 residue (Fallon et al., 2006). In line with data by other groups (Murata et al., 2011), our data indicate that PINK1 promotes AKT phosphorylation on S473 residue through mTORC2 pathway. Therefore, our model suggests that Parkin regulates AKT phosphorylation on T308 residue whereas; PINK1 regulates AKT phosphorylation on S473 residue, which potentially enhances phosphorylation of T308 residue by Parkin. However, in the absence of PINK1 or in the presence of Rapamycin, Parkin is not capable to enhance AKT phosphorylation to induce maximum activity. In other words, the regulatory function of PINK1 on mTORC2 is necessary for the sequential activation of the pathway by Parkin.

Figure 4.1. Model: PINK1, Parkin and AKT pro-survival pathway. AKT has two phosphorylation sites; T308 and S473. mTORC2 promotes AKT phosphorylation on S473 residue. PI3K/PDK1 directly phosphorylates AKT on T308 residue. PINK1 enhances AKT phosphorylation by regulating the mTORC2 pathway. Parkin induces AKT phosphorylation through the PI3K/PDK1/AKT pathway. Phosphorylation of the S473 residue through the PINK1/mTORC2 pathway primes for phosphorylation of the T308 residue through the Parkin/ PI3K/PDK1 pathway which upregulates AKT activity. The regulatory function of PINK1 on mTORC2 is necessary for the activation of the pathway. In the absence of PINK1 or by blocking the mTOR pathway, the AKT pathway does not reach to its maximum activity by the Parkin/PI3K/PDK1 pathway.

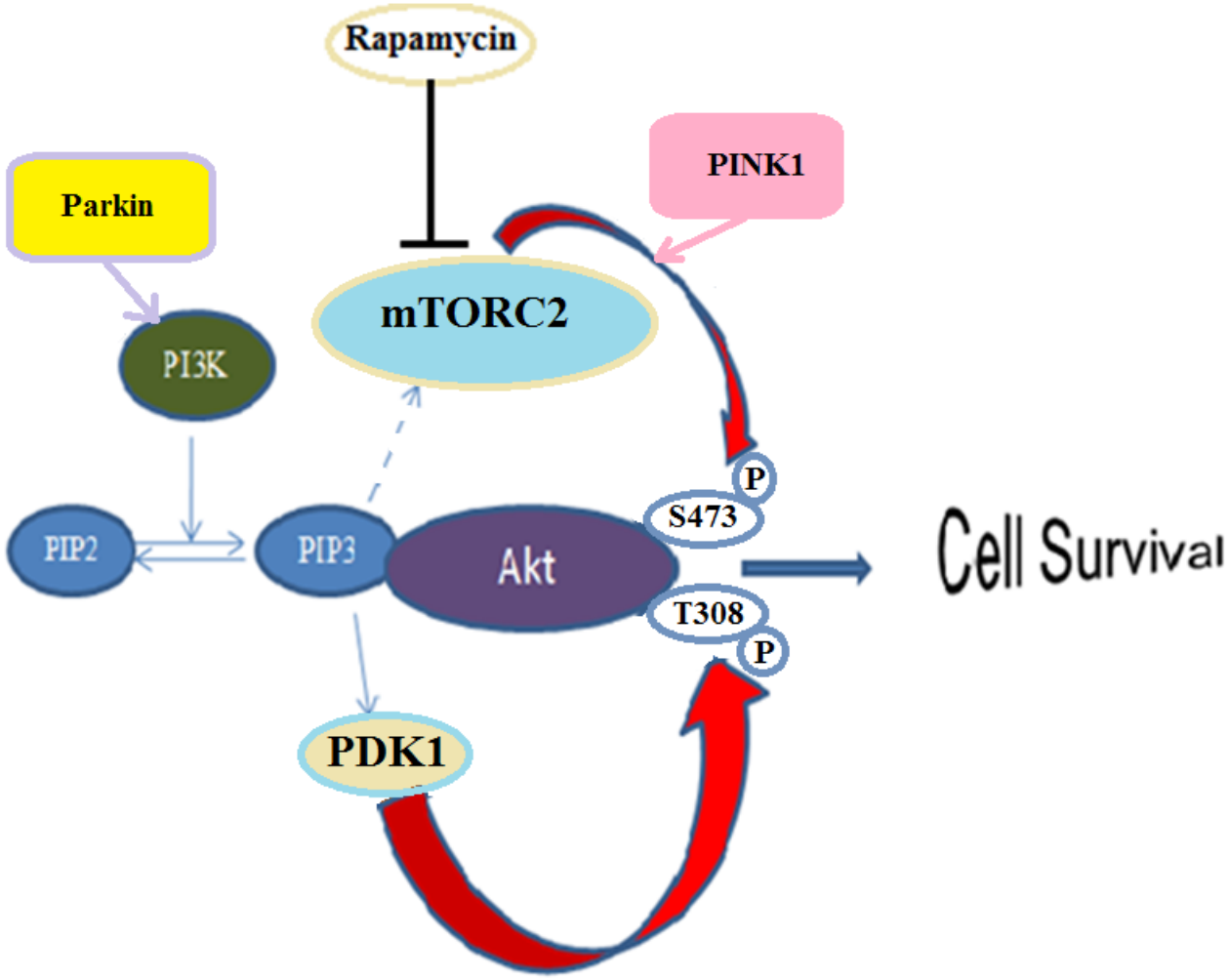

Rapamycin
PINK1
Parkin
mTORC2
PI3K
P
S473
PIP2
PIP3
Akt
Cell Survival
T308
P
PDK1

Figure 4.1

Collectively, based on the evidence presented as part of this thesis, we have introduced the AKT pathway, a major neuronal pro-survival pathway induced upon oxidative stress, as a potential mediator for the neuroprotective functions of PINK1 and Parkin. Additionally, our data demonstrates that the AKT/mTOR pathway, at least in part, serves as a potential cytosolic target for PINK1. We have also proposed that Parkin potentially regulates AKT through a pathway (most likely PI3K/AKT pathway) distinct of the mTOR pathway. Future studies will more carefully examine if PI3K pathway blockers could inhibit the neuroprotective effect of Parkin. It is also important to examine if induction of PI3K pathway can rescue the sensitivity of Parkin deficient neurons to ischemic insults. Additionally, it would be interesting, in the reverse experimental paradigms, to examine if PINK1 can rescue the effect of Parkin deficiency on neuronal survival in the presence of the AKT pathway blockers. Since phosphorylation of T308 residue is important for full activation of AKT pathway, assessment of AKT phosphorylation on this residue using our experimental paradigms, would be very informative in the future studies. In this regard, application of PI3K/AKT inhibitors alone or in combination with Rapamycin would potentially help to collect valuable data to address the interconnection of these two converging pathways as suggested by the model presented in **Figure 4.1**.

Lastly yet importantly, it would be very critical to validate our *in vitro* data using *in vivo* experimental settings.

There is growing evidence suggesting that, under basal conditions, PINK1 is cleaved by multiple mitochondrial proteases such as MPP, PARL and AFG3L2 (Greene et al., 2012; Jin et al., 2010). After cleavage, PINK1 is believed to be released into the cytosol where it is subjected to proteasomal degradation. Despite a growing body of evidence supporting this model, the functionality of cleaved PINK1 is still the subject of much debate. In fact, the current knowledge of the field does not rule out the possibility that cleaved PINK1 might exit mitochondria to regulate targets in the cytosol. Therefore, it would be interesting to address if these cleaved forms are capable of activating the AKT pathway in our experimental paradigms. These approaches are particularly important given that our data from the present and previous studies indicate that cytosolic PINK1 [PINK1 with deleted mitochondrial motif (ΔN)] is protective against both glutamate treatment and mitochondrial neurotoxin, MPTP (Haque et al., 2008).

Work from our lab and others have indicated that PINK1, Parkin and DJ-1 functionally interact to regulate mitochondrial function and promote neuronal survival (Haque et al., 2012; Irrcher et al., 2010; Thomas et al., 2011). Additionally, we have shown that DJ-1 modulates the AKT pathway to protect the nigrostriatal axis from the neurotoxin MPTP (Aleyasin et al., 2010). These observations together with my current data provide a strong basis for future studies to further elucidate the interconnection of these three PD genes and the AKT pathway. For instance, in a previous study, we noted that DJ-1 confers a neuroprotective role against *in vitro* and *in vivo* ischemic insult (Aleyasin et al., 2007). Considering functional interaction of PINK1 and Parkin with DJ-1, it would be interesting to examine if DJ-1 can rescue the neuronal sensitivity of PINK1 or Parkin deficient neurons

to ischemic insults. Conversely, we need to examine whether PINK1 or Parkin can rescue the effect of DJ-1 deficiency on neuronal survival in our ischemic experimental paradigms. Additionally, it would be interesting, to examine if DJ-1 can rescue the effect of PINK1 or Parkin deficiency on neuronal survival in the presence of the AKT pathway blockers. In the reverse experimental paradigms, we need to examine if AKT can potentially mediate the pro-survival effect of PINK1 or Parkin in the absence of DJ-1.

4.3. Conclusion:

Based on the evidence presented as part of this thesis, we propose that PINK1 and Parkin are involved in fine-tuning the neuronal survival responses in ischemic context. Our data also suggests a cytosolic role for PINK1 and Parkin in AKT mediated neuroprotection in a rodent model of ischemia. In addition, our study suggests that PINK1 acts upstream of AKT, thereby facilitating its activation through the mTOR pathway following neuronal injury induced by excitotoxicity.

Taken together, this experimental setting established a platform to study the neuroprotective function of PINK1 and Parkin in more acute brain injury such as ischemia, with the opportunity to investigate these genes in a more clinically relevant manner. Our findings strengthen the current body of knowledge supporting genetic and biochemical interaction between PINK1, Parkin and various cellular processes with a great potential to be employed in future translational approaches, as promising targets for developing novel therapeutic for stroke and PD.

Appendix I:

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Bag2 mediated regulation of Pink1 is critical for mitochondrial translocation of Parkin and neuronal survival*

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*Running title: *Bag2 stabilizes Pink1 to allow for proper mitochondrial translocation of Parkin*

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Key words: Parkinson's disease, Bag2, Pink1, mitochondria, translocation, neuronal death

Abstract

Emerging evidence has demonstrated a growing genetic component in Parkinson's disease (PD). For instance, loss of function mutations in *Pink1* or *Parkin* can cause autosomal recessive PD. Recently, Pink1 and Parkin have been implicated in the same signaling pathway to regulate mitochondrial clearance through recruitment of Parkin by stabilization of Pink1 on the outer membrane of depolarized mitochondria. The precise mechanisms that govern this process remain enigmatic. In this study, we identify Bcl2-associated athanogene 2 (Bag2) as a factor that promotes mitophagy. Bag2 inhibits Pink1 degradation by blocking the ubiquitinylation pathway. Stabilization of Pink1 by Bag2 triggers Parkin-mediated mitophagy and protects neurons against MPP^+-induced oxidative stress in an *in vitro* cell model of PD. Collectively, our findings support the notion that Bag2 is an upstream regulator of the Pink1/Parkin signaling pathway.

Parkinson's disease (PD) is the second most common neurodegenerative disorder characterized by the selective loss of the pigmented dopaminergic neurons of the substantia nigra pars compacta (SNc) (1). Although most of PD cases are sporadic in nature (2), mutations in several genes have been linked to familial forms of PD (reviewed in (3)). Indeed, these familial genes serve as important vehicles to study potential mechanisms of pathogenesis in PD. In this regard, increasing evidence suggests that mitochondrial dysfunction may play a critical role in both inherited and sporadic forms of PD, although the precise role of mitochondrial dysfunction in PD is unclear (4). Recently, two familial recessive PD genes, (PTEN)-induced putative kinase-1 (*Pink1*), a mitochondria-localized serine–threonine kinase gene and *Parkin*, an E3 ubiquitin ligase gene, have been identified as acting along similar pathways in regulating mitochondrial quality control in mammalian systems (5-8). These findings are supported by genetic studies in *Drosophila* models of PD, which show that Parkin and Pink1 function in a common pathway, with Parkin being a downstream player of Pink1 (9-11). A third recessive PD gene *DJ-1* associated with regulation of oxidative stress also regulates Pink1/Parkin-mediated control of mitochondrial health (12).

Pink1 is normally shuttled to the inner mitochondrial membrane where it is processed by multiple proteases (13). The endogenous role of Pink1 at this site is unknown. However, *Pink1* deficient cells display altered calcium homeostasis at the mitochondria as well as altered mitochondrial function (14). Processed Pink1 at the inner mitochondrial membrane is also proposed to be shuttled to the cytosol where it is

degraded by the proteasome (15). However, under conditions of mitochondrial stress such as with the treatment of the mitochondrial uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP) or with the complex I inhibitor 1-methyl-4-phenylpyridinium (MPP^+), Pink1 can also regulate mitophagy in a Parkin dependent fashion (12,16-19). The framework by which this mitochondrial control pathway occurs has been generally delineated. Pink1 is stabilized on the outer membranes of damaged/depolarized mitochondria (8). Pink1, then recruits Parkin to the mitochondria where it ubiquitinylates a number of substrates including Mfn1, Mfn2, VDAC1 and Mcl1 which in turn activates mitophagy (8,16-22). More recently, recruitment of Parkin by Pink1 to mitochondria has also been observed in primary neurons in a reactive oxygen species (ROS)-dependent manner (12,23). Interestingly, processed cytosolic Pink1 may also interact with cytosolic Parkin, limiting its translocation to the mitochondria and mitophagy (15). Likewise, in the presence of MG-132, an inhibitor of proteasome, the level of processed cytosolic Pink1 is significantly increased (24), indicating that turnover of Pink1 is through the proteasomal pathway. Consistent with this, Pink1 has also been shown to be ubiquitinylated (25,26). Interestingly, a recent report indicated that Bag2 can regulate the ubiquitinylation of exogenous Pink1 in HEK293 cells (26). Taken together, these findings indicate that the regulation of Pink1 levels and activity is crucial to mitochondrial function and quality at a number of levels including regulation of mitochondrial health. The exact mechanism through which Pink1 activity/levels is governed is not fully understood. In addition, the biological consequences of dysregulation of Pink1 processing by ubiquitin-mediated pathways are unclear. In this study, we examined the role of Bag2 and provide evidence that it physically interacts with Pink1 *in vivo* and stabilizes Pink1 from proteasomal degradation, facilitates recruitment of Parkin to depolarized mitochondria and protects primary cortical neurons under stress conditions in a Pink1-dependent manner.

Experimental procedures

Mice-Germ line-deleted *Pink1* and *Parkin* mice have previously been described in detail, respectively (27,28). CD1 mice were obtained from Charles River Laboratories, Canada. All animal procedures were approved by the University of Ottawa Animal Care Committee, and animals were maintained in strict accordance with the Guidelines for the Use and Treatment of Animals put forth by the Animal Care Council of Canada and endorsed by the Canadian Institutes of Health Research.

Antibodies-The following antibodies were used: rabbit anti-Bag2 (Abcam), rabbit anti-Pink1(494) (Novus), mouse anti-Flag (Sigma), mouse anti-myc and rabbit anti-Raf-1 (Santa Cruz), mouse anti-V5 and anti-complex I (Invitrogen), mouse anti-Actin (Sigma), rabbit anti-TOM20 (Santa Cruz), mouse anti-ubiquitin (Abcam), anti-mouse and anti-rabbit horse radish peroxidase-conjugated secondary antibody (eBiosciences) for IP Western blots and anti-mouse and anti-rabbit horse radish peroxidase-conjugated secondary antibodies (Millipore) for regular Western blots. The secondary antibodies labeled by Alexa Fluor®564 for mouse IgG or Alexa Fluor®633 for rabbit IgG were purchased from Life Technologies. All primary antibodies were used at 1:5,000 dilution except anti-actin which 1:50,000 for Western blot analyses. All of the primary antibodies were diluted 1:200 for immunofluorescent (IF) staining. The secondary antibodies for IP were diluted 1:5,000. The secondary antibodies for Western blots were diluted 1:10,000. The secondary antibodies for IF staining were diluted 1:200.

Constructs- *Pink1-V5* was a gift from Dr. Mark Cookson. *Myc-Bag2* construct was obtained from Dr. Suneil K. Kalia as a kindly gift. *GFP-Parkin* in pEGFP vector was gifted from Dr. Edward Fon. *Pink1-Flag* and *Pink1(K219M)-Flag* were inserted into pAdTrack vector by using NotI and HindIII. *CHIP* was cloned into pCMV-3Tag vector by using BamHI and XhoI. *HA-HSP90* construct was a gift from William Sessa (Addgene plasmid # 22487).

Cell culture-HEK293T cells were cultured with 10% fetal bovine serum (Sigma) in Dulbecco's modified Eagle's media (Sigma). Cortical neurons were dissected from E14.5–15.5 WT CD1, *Pink1* (C57BL/6) or *Parkin* C57BL/6 mice. The primary cortical neurons were maintained in Neurobasal media (Invitrogen) supplemented with B27 with

antioxidants (Invitrogen), N2 (Invitrogen), 0.5 mM L-glutamine (Sigma) and penicillin/streptomycin (Invitrogen) as previously described (29,30).

Proteomic screen-HEK293T cells were transiently transfected with *Pink1-Flag* plasmid. The cells were then cultured in fresh medium (Dulbecco's modified Eagle's medium (DMEM) with10% fetal bovine serum (FBS)) for 24 hours. The Cells were then harvested and lysed by lysis buffer (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 10 µg/ml aprotinin, 0.2 mM AEBSF (Calbiochem)), and cleared from cell debris by centrifugation at 20,000 *g* for 30 min. The cleared cell lysate was subjected to immunoprecipitation using M2-Agarose resin (Sigma-Aldrich) for 1 hour. After 3 time washes, the co-precipitated proteins were eluted by 50 mM ammonium bicarbonate, containing 400 µM Flag peptide. The purified proteins were subjected to SDS-PAGE, detected by colloidal Coomassie staining, and protein bands from qualified lanes were excised from the gel. These proteins were treated with DTT, iodoacetamide (to alkylate the free sulfhydryl groups) and trypsin, and the digested peptides were then purified from the gel and concentrated and analyzed by mass spectrometry. As reported earlier (31), the data was generated using an LCQ Deca mass spectrometer (Thermo Finnigan). Mascot version 1.9 (Matrix Sciences, www.matrixscience.com) was used to analyze the obtained spectra by searching against a human protein sequence database with 122989 entries. The settings to run the Mascot were as follows: search mode: MS/MS Ion, fixed modification: carbamidomethyl on cysteine, variable modification: oxidation on methionine, peptide mass tolerance: 2 Da, fragment mass tolerance: 0.4 Da, maximum missed cleavages: 2, enzyme: trypsin. The Mascot score is the probability of randomness of the match, and is reported as $-10\mathrm{LOG}_{10}(P)$, where P is the absolute probability. In other word, the score of 30 means the absolute probability of 10^{-3}.

Assay for Mitochondrial translocation of Parkin and survival-For study of Parkin translocation in HEK293T cells, 2.0×10^5 cells/well were transfected with plasmids by Lipofectamine 2000 as manufacture's instruction for suspended cells in 24-well plate. Briefly, *GFP-Parkin* was transfected with either the empty vector or *myc-Bag2* at a ratio of 1:3 (0.5 µg total) with 1 µl of Lipofectamine 2000 (Invitrogen). 24 hours after transfection, the cells were treated with 10 µM CCCP (Sigma) for up to 2 hours to induce oxidative stress as previously described (12). For Parkin translocation in neurons, the dissected neurons were seeded on Poly-D-Lysine-coated coverslips in 24-well plates at a density of 1.5×10^5 cells per well. 3 days after plating, the neurons were transfected with plasmids, *GFP-Parkin* with empty vector or *GFP-Parkin* with *myc-Bag2* at a ratio of 1:3 (0.5 µg total) with 1 µl of of Lipofectamine 3000 (Invitrogen). 24 hours after transfection, the neurons were treated with 10 µM CCCP (Sigma) for 2 hours to depolarize mitochondrial membrane potential as previously described (12). After treatment, cells were fixed, permeabilized and immunostained with the indicated antibodies and visualized by confocal microscopy.

Cell fractionation-The mitochondrial and cytosolic fractions were isolated as previously described (13). Briefly, collected cells from one 10 cm culture dish were resuspended in 1 ml of BIB (210 mM mannitol, 70 mM sucrose, 5 mM Tris pH 7.4, 0.2 mM EGTA, 0.1 mM EDTA, 0.1% bovine serum albumin and cocktail protease inhibitors) and homogenized with 50 strokes using a 2 ml glass homogenizer. The supernatant, from 2 times centrifugations with 1000g for 10 minutes at 4ºC, was subjected to centrifugation with 12,000g for 10 minutes at 4ºC to pellet mitochondria. The supernatant was used as cytosolic fraction. The mitochondrial pellet was resuspended in 1 ml of BIB buffer for sonication to extract mitochondrial proteins. The extracts were subjected to IP.

Survival assays-For plasmids transfection, 3 days after plating, the neurons were transfected. 24 hours after transfection, the neurons were treated with 20 µM MPP^+ for 48 hours and assessed for survival as previously described (30) Briefly, following fixation, GFP positive neurons were assessed for nuclear integrity as determined by Hoechst staining. Neurons with punctate or condensed nuclei were assessed as dead. For siRNAs transfection, neurons were transfected with 30 pmol/well (24-well plate) siRNAs to mouse *Bag2*, *Pink1* or *Parkin* plus 30 pmol/well of control siRNA to 60 pmol siRNA in total. For co-transfection of siRNAs, neurons were transfected

with 30 pmol/well for each using Lipofectamine 2000 as previously described (32). 24 hours after transfection, the neurons were treated with 20 µM MPP^+ for 48 hours. The MTT assay was carried out. Briefly, the neurons were incubated with 10 µl of 5mg/ml MTT for 4 hours. After removal of the medium with MTT, 100 µl of solubilization buffer was applied for extracting the converted MTT for 2 hours. The plate was read at 570 nm by a plate reader (30).

Determination of Pink1 levels and ubiquitinylation-For determination of levels of overexpressed Pink1 , 0.1 µg of *Pink1-Flag* was transfected with 0.4 µg of empty vector or *myc-Bag2* using 1 µl of Lipofectamine 2000 (Invitrogen) onto 2.0×10^5 HEK293T cells/well in 24-well plates. 2 day after transfection, the total cell lysates were collected and subjected to Western blot analysis with anti-Flag antibody for Pink1 and anti-myc antibody for myc-Bag2. For endogenous Pink1, empty vector or *myc-Bag2* was transfected into HEK293T cells for 2 days, the total cell lysates were analyzed by Western blot for Pink1. For the ubiquitinylation assay of overexpressed Pink1, 3.0×10^6 HEK293T cells in 35 mm dish were transfected with 1 µg *Pink1-Flag* in the presence of 3 µg empty vector or *myc-Bag2* utilizing 8 µl of Lipofectamine 2000. 2 days after transfection, the total proteins were extracted in 200 µl of RIPA buffer. After 10 minutes boiling and 20 minutes centrifugation (20,000g) at 4°C, the supernatants were diluted 2 times with water for IP using 1 µg of anti-Flag antibody and 30 µl of anti-mouse IgG beads (eBiosciences) for 2 hours at 4°C. The beads were washed for 4 times using a wash buffer containing 20 mM Tris at pH7.4, 300 mM NaCl, 0.1% SDS and 1 mM EDTA. Proteins were eluted in 2×SDS sample buffer for separation by 4-15% SDS-PAGE gel (Biorad) and Western blot analyses utilizing an anti-ubiquitin antibody. The ubiquitinylation assay of endogenous Pink1 was performed similar to that described above with the exception that HEK293T cells were transfected with empty vector or *myc-Bag2* and the IP was carried out with anti-Pink1 antibody.

siRNA-All siRNAs were purchased from Santa Cruz Biotechnology. For siRNA transfection, 30 pmol of siRNA/well were transfected with 1 µl of Lipofectamine 2000 for 24 wells and 150 pmol of siRNA were transfected with 8 µl of Lipofectamine 2000 for a 35 mm dish. Twenty-four hours after siRNA transfection, plasmids were transfected as above described for protein level assay or ubiquitinylation level assay.

Statistical analysis-Statistical significance was determined using paired Student's *t*-test for related samples unless otherwise stated. Two-way ANOVA was used in Parkin translocation and Bag2 regulated survival to determine significance between WT and KO in control and treated conditions. All data are presented as mean ± standard error of the mean (SEM). Significance at $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$; no significance is denoted by N.S.

Results

Physical interaction between Pink1 and Bag2

Pink1 plays an important role in regulating dopaminergic loss (5) as well as a number of critical biological processes such as mitochondrial quality control. However, the potentially diverse set of processes which regulate Pink1 function or its downstream effectors are not completely known. In particular the mechanisms which control Pink1 levels and stability, a likely important component of Pink1 regulation, are not completely clear. To identify these regulatory mechanisms, we performed a Pink1 interactomic screen to search for proteins associated with Pink1 based on mass spectrometric strategy (31). One particular target of our interest in regulation of Pink1 levels was Bag2, which was identified as an interactor of Pink1 with a Mascot Protein Score of 99.

Bag2 has been previously identified as a chaperone protein involved in proteasomal process (33), and we hypothesized that it might be critical for regulation of Pink1 stability. Accordingly, we pursued this target in more detail. To confirm the physical interaction, we first co-expressed *V5* tagged *Pink1* (*Pink1-V5*) with *myc* tagged *Bag2* (*myc-Bag2*) in HEK293T cells. Cell extracts were then immunoprecipitated with IgG control or myc antibody and probed by Western blot analyses for V5 to assess Bag2 interaction. As shown in Fig 1A (left panel) interaction was observed with myc pulldown but not IgG control. We also performed the reverse interaction studies by immunoprecipitating for V5 and probing by Western analyses for myc. Consistent with our initial results, an interaction between expressed

Bag2 and Pink1 was also observed (the right panel in Fig. 1A). As shown in Fig. 1A, Bag2 interacts with not only full length Pink1 but also the processed Pink1. Previous studies indicated that cleavage of Pink1 is processed in mitochondria (13). Therefore, the mitochondrial and cytosolic fractions from HEK293T cells transfected with *Pink1-Flag* and *myc-Bag2* were subjected to immunoprecipitation with myc antibody to check where the interaction of Pink1 and Bag2 takes place. As shown in Fig. 1B, the interaction of Pink1 and Bag2 was observed in both fractions but predominantly in the mitochondrial fraction. Finally, to ensure that this interaction could also be observed with endogenous Pink1 and Bag2, we probed non-transfected HEK 293T cells extract by immunoprecipitating with Pink1 antibody or IgG control and immunoblotting with Bag2 antibody. HEK293T cells were utilized to study endogenous interaction since we can readily detect endogenous Pink1 with available antibodies. This interaction could not be studied in primary neurons since Pink1 antibodies do not readily recognize Pink1 from murine or rat sources. In agreement with our tagged expression interaction studies, we detected endogenous interaction between Pink1 and Bag2 confirming the validity of this interaction (Fig. 1C).

Bag2 modulates Pink1 levels through proteasomal degradation

To explore the functional consequences of this interaction, we first investigated whether Pink1 level is regulated by Bag2. We initially explored the effects of expression of Bag2 on exogenously expressed tagged Pink1 levels. As shown in Fig. 2A, increasing amounts of *myc-Bag2* plasmid (balanced for amounts by a control vector) was cotransfected with a constant level of *Pink1* plasmid. Increasing expression of Bag2 was confirmed by Western blot analyses. We showed that increased Bag2 expression correlates with increased levels of Flag tagged Pink1. Recent studies suggest that chaperones such as HSP90 also affect Pink stability (34,35). We accordingly checked whether expression of HSP90 may affect Pink1 stability in ways similar to that of Bag2 expression. We found that HSP90 failed to affect Pink1 levels suggesting that Bag2 acts in ways different than that of HSP90 (Fig. 2B). We next performed the converse experiment where we examined siRNA-mediated downregulation of endogenous Bag2 on the levels of exogenous Pink1. As shown in the right panel of Fig. 2A, siBag2 when compared to scrambled siRNA controls led to significantly reduced levels of Flag-tagged Pink1. siRNA mediated downregulation of Bag2 was confirmed by Western blot analyses. Finally, we examined whether overexpression of Bag2 or siRNA-mediated knockdown of Bag2 affects endogenous Pink1 level in HEK293T cells. Consistent with the effect of Bag2 on exogenous Pink1, expression of Bag2 increased levels of Pink1 while siRNA-mediated downregulation of Bag2 reduced endogenous Pink1 levels (Fig. 2C).

Next, we explored whether the effect of Bag2 on Pink1 protein levels is mediated via a proteasomal pathway. HEK293T cells were cotransfected with *Flag* tagged *Pink1* or *Flag* tagged *Pink1(K219M)*, a kinase dead mutant of *Pink1*, along with *myc-Bag2*, or, *Pink1* and siRNA for *Bag2* and treated with the proteasomal inhibitor, MG132 to stabilize ubiquitinylated Pink1. After immunoprecipitation of Pink1 with anti-Flag antibody, ubiquitinylated Pink1 was detected by Western blot analyses using an anti-ubiquitin antibody. As shown in the left panel of Fig. 3A, overexpression of Bag2 dramatically reduced the level of ubiquitinylated Pink1. In this experiment, it is interesting to note that MG132 treatment only slightly elevated the levels of ubiquitinylated Pink1-mostly higher molecular weight species. This may be due to the differential kinetics of turnover of ubiquitinylated Pink1 species. Similarly, we also observed that Bag2 dramatically affects the level of ubiquitinylated Pink1(K219M) in HEK293T cells along with overexpression of Bag2 as shown in the middle panel of Fig. 3A. In contrast, knockdown of Bag2 led to the converse and increased Pink1 ubiquitinylation as shown in the right panel of Fig. 3A. In agreement with the effect of Bag2 on exogenously expressed Pink1, overexpression or knockdown of Bag2 decreased or increased the ubiquitinylated level of endogenous Pink1, respectively (Fig. 3B). Importantly, Bag2 expression did not significantly affect levels of total ubiquitinylation. Bag2 has been associated with CHIP E3 ubiquitinylation activity. Previous results showed that Bag2 inhibits CHIP E3 ligase activity (36). Accordingly, we examined whether CHIP may be involved in Bag2-mediated effects on ubiquitinylation of Pink1. Expression of CHIP with exogenous Pink1 unexpectedly led to an

increase in total Pink1 and a decrease in ubiquitinylated Pink1 (the left panels of Fig. 3C). Conversely, knockdown of CHIP resulted in a decrease in total Pink1 but an increase in ubiquitinylated Pink1 (the right panels of Fig. 3C). While not completely conclusive, this suggests a CHIP independent pathway of regulation of Pink1 by Bag2. Increasing evidence suggests that Pink1 and Parkin function in the same pathway in regulating mitochondrial functions (9-11,37,38). Hence, we examined whether Bag2 affects the level of Parkin. Expression of Bag2 did not cause increase of Parkin in HEK293T cells (Fig. 3D), indicating Bag2 may regulate Parkin mitochondrial translocation through Pink1.

Bag2 regulates Parkin translocation to mitochondria in a Pink1-dependent manner

We next examined whether Bag2-mediated regulation of Pink1 affects known Pink1 relevant biological outcomes. Loss of mitochondrial membrane potential promotes Pink1/Parkin mediated mitophagy in different cell lines as well as primary cultured neurons (8,12,16,18,19). To test whether the stabilizing effect of Bag2 exerts on Pink1 translates into a functional outcome, we determined whether Bag2 influences Parkin translocation in a Pink1-dependent manner. To this end, HEK293T cells were cotransfected with *GFP* or *GFP-Parkin* along with *myc-Bag2* or siRNA for *Bag2*. 1-2 days after transfection, cells were treated with CCCP to induce mitochondrial depolarization. As shown in Fig. 4A and B, expression of exogenous Bag2 significantly increased mitochondrial translocation of Parkin when compared to controls transfected with control vector. Importantly, downregulation of Bag2 almost significantly diminished Parkin translocation to mitochondria (Fig. 4C and D). In agreement with the results from immunofluorescent assay, the biochemical fractionation studies confirmed that Bag2 significantly affects the mitochondrial translocation levels of overexpressed or endogenous Parkin (Fig. 4E and F).

Since mitochondrial translocation of Parkin was also recently observed in primary neurons (12,23), we investigated whether Bag2 also affects mitochondrial translocation of Parkin in primary neurons after mitochondrial depolarization. We found that Bag2 expression significantly increased mitochondrial translocation of Parkin induced by CCCP. The Bag2-mediated increase in Parkin translocation was completely dependent upon Pink1 since Parkin translocation did not occur in Pink1 deficient neurons (Fig. 5). As an alternative control, we also examined whether Bag2 might affect levels of Parkin as an alternative explanation of the observed increased in Parkin translocation. However, expression level of Bag2 appeared to have no effect on Parkin levels (Fig. 3D and the top panel of Fig. 4D). Taken together this suggests a model by which Bag2 contributes to increased Parkin levels on mitochondria through a Pink1-mediated pathway which is independent of any global effect on Parkin levels.

Bag2 protects neuronal death through a Pink1/Parkin pathway

Both Pink1 and Parkin are also reported to promote survival of neurons in response to multiple stresses. Given that overexpression of Pink1 protects cells against ROS induced death (5,39) and Bag2 promotes Parkin translocation through Pink1, we investigated whether Bag2 protects neurons against oxidative stress induced by MPP^+ through the Pink1/Parkin signaling cascade. First, we evaluated whether Bag2 itself protects cultured primary cortical neuron in an MPP^+-induced cell model of PD. As shown in the top panel of Fig. 6A, overexpression of Bag2 protected neurons against MPP^+-induced neuronal death. We reported previously that acute downregulation of Pink1 sensitizes neurons to MPP^+ (39). We recapitulate this observation here and also confirm the same with Parkin downregulation. Interestingly, siRNA-mediated downregulation of Bag2 led to the same degree of sensitization to death observed with downregulation of either Parkin or Pink1. Furthermore, combined downregulation of Bag2 and Pink1 or Parkin did not further increase death when compared to either alone. This was also true of co-treatment with siRNA to both Parkin and Pink1 (Fig. 6A bottom panel). We also assessed whether the survival effects of Bag2 expression was dependent upon Pink1 or Parkin. We utilized *Pink1* or *Parkin* deficient neurons obtained from germ line KO animals. In this case, sensitization of death induced by loss of *Pink1* or *Parkin* alone is not observed as shown with acute knockdown. This may be due to compensation resulting from long term *Pink1*/*Parkin* loss. Indeed, *Pink1*/*Parkin*

KO animals do not show dopaminergic loss (40,41). Importantly, however, protection observed in WT cultures with Bag2 expression is abolished with either *Parkin* or *Pink1* deficiency (Fig 6B). Taken together, this data suggests that Bag2 can increase neuronal viability through a Pink1/Parkin pathway.

Discussion

Previous work has shown that Pink1 is degraded by the proteasomal pathway (42,43). After being processed by a number of proteases, Pink1 is degraded by the proteasome (13,44-46), resulting in a low abundance of Pink1 in healthy cells. Upon the loss of mitochondrial membrane potential, accumulated full-length Pink1 (FL-Pink1) on the outer mitochondrial membrane recruits Parkin to mitochondria, leading to mitochondrial clearance via autophagy (16-19). Some reports have also suggested that cleaved Pink1 can also perform the same function. (46,47). These observations indicate that regulation of Pink1 processing and turnover is critical to its biological function. Presently, we identify Bag2 as one mechanism by which Pink1 levels are regulated and link its effect on Pink1 with its downstream biological effects.

Bag2 is one member of the Bag family. Its members are reported to have a diverse range of effects, particularly on stimulating or interfering with ubiquitin-E3 ligase activity (36,48). For example, Bag1 stimulates chaperone-associated E3-ubiquitin ligase activity, facilitating proteasomal activity for protein degradation. Bag2 inhibits chaperone-associated E3-ubiquitin ligase activity, interrupting protein degradation (36,48,49). Beyond regulation of chaperone-associated E3-ubiquitin ligase activity, Bag5 also inhibits the E3 ligase activity of Parkin through its functional interaction with Parkin (50).

We provide evidence that Bag2 is central to regulation of Pink1 stability. First, Bag2 expression increases levels of both exogenously expressed and endogenous Pink1. Second, Bag2 expression reduces ubiquitinylation of expressed or endogenous Pink1. These results are consistent with a recent report that expression of Bag2 reduces ubiquitinylated Pink1 possibly through blocking ubiquitin ligase activity (26). Importantly, we also show that downregulation of endogenous Bag2 leads to the converse observations providing much clearer evidence that Bag2 plays a central role in regulating Pink1 levels. It is interesting that Bag2 appears to regulate stability of full length as well as processed forms of Pink1. This is in contrast to MG132 treatment, which stabilizes only the more processed forms. The reason for this is unclear but Bag2 does interact with both forms of Pink1. We can only speculate that the interaction of Bag2 with full length Pink1 may slow down conversion of the full length Pink1 to processed Pink1 by proteases such as Parl (45).

Given the evidence that Bag2 can regulate proteasomal activity through inhibition of CHIP E3 ligase activity, we anticipated that ubiquitinylated level of Pink1 should be up-regulated upon expression of CHIP. However, we observed a decrease in ubiquitinylated Pink1 and an increase in Pink1 levels as a result of CHIP expression. Furthermore, we found that HSP90, a chaperone protein important for CHIP-mediated proteasomal degradation (51), did not influence the level of Pink1, further indicating that CHIP-mediated protein degradation may not have a critical role in Pink degradation. Taken together, these results suggest that an alternative E3 ligase other than CHIP is a target for Bag2.

Importantly, we also assessed the biological consequences of Bag2 expression. We first assessed the effects of Bag2 on the Parkin mediated mitochondrial control pathway. Given that accumulation of Pink1 recruits Parkin to the outer membrane of mitochondria (8,16,18,19), the stabilization of Pink1 should augment mitochondrial translocation of Parkin upon depolarization of mitochondria. Indeed, expression of Bag2 significantly increased Parkin translocation to the mitochondria. In addition, downregulation of Bag2 by siRNA led to the converse observation, dramatically blocking Parkin recruitment to the mitochondria. Importantly, we also assessed whether Bag2 might also affect Parkin levels thereby explaining increased Parkin translocation observed at the mitochondria. However, expression of Bag2 had no effect on Parkin levels suggesting that Bag2 did not affect the mitochondrial control pathway through direct regulation of Parkin levels. Finally, we also determined whether Bag2 might affect Parkin translocation through a pathway unrelated to Pink1. In this regard, we assessed whether Bag2 might increase Parkin translocation in the absence

of Pink1. However, Bag2 did not increase Parkin translocation in Pink1 KO neurons. Taken together, our results indicate that Bag2 increases Pink1 levels which lead to increased Parkin translocation, an important reported step in mitochondrial quality control.

We also assessed whether/how Bag2 might affect neuronal survival in the presence of mitochondrial stress. We demonstrate that Bag2 expression is protective in this model whereby downregulation of Bag2 sensitizes neurons to death. Importantly, we showed that the protective effect is completely dependent upon the presence of either Parkin or Pink1. This latter observation is consistent with the notion that both Pink1 and Parkin have a protective effect in neurons against mitochondria and oxidative stress (11,39,52). While our data indicate that the protective effects of Bag2 rely on Pink1/Parkin, it is important to note that we have not established that mitochondrial quality control is the mechanism by which Bag2 regulates survival. Indeed, some data has indicated that Parkin regulated mitophagy does not occur in the presence of mitochondrial damage induced by loss of TFAM (53). However, Parkin/Pink1 mediated pathways of mitochondrial quality control have been noted in neurons upon oxidative/mitochondrial stress (12,23). Regardless, our data indicates that Bag2 regulates a protective response through a Pink1/Parkin mediated pathway. We propose a model by which this occurs through an increase in Pink1 levels.

Finally, previous work indicated that import of the FL-Pink1 into mitochondria is required for processing of FL-Pink1 to cytosolic Pink1 by different proteases (13,44-46,54). The constitutive turnover of Pink1 is presumably a critical regulatory machinery to inhibit Pink1/Parkin pathway in healthy cells. On the contrary, FL-Pink1 is accumulated on the outer surface of depolarized mitochondria to trigger the activation of the Pink1/Parkin pathway (reviewed in (55)). Our findings demonstrate that Bag2 expression results in the accumulation of different forms of Pink1, especially FL-Pink1, suggesting that Bag2 may block the import of FL-Pink1 into mitochondria, thus resulting in the accumulation of FL-Pink1. Consistent with this model, we observed more mitochondrial translocation of Parkin in cells with expression of Bag2 even under conditions lacking mitochondrial stress. We propose two possibilities by which Bag2 manipulates Pink1 level. First, Bag2 may prevent Pink1 degradation by inhibiting the proteasomal pathway; second, Bag2 interrupts the import of Pink1 to mitochondria, which would result in inhibition of Pink1 processing and increased FL-Pink1. It will be important to distinguish between these possibilities in future studies.

Conflict of interest

The authors declare that there are no conflicts of interests with the contents of the article.

The author contributions

DQ, RSS and DSP designed the study. DQ and DSP wrote the paper. DQ, AH and KD performed the majority of experiments. EH, AJ, PCM, MWCR, DSI, FS, SJH, TH and SC provided technical assistants. DD and DF designed and preformed proteomic screen. All authors reviewed the results and approved the final version of the manuscript.

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Figure legends

Fig. 1. Bag2 forms a complex with Pink1. A. Bag2 interacts with Pink1 upon expression of Pink1 and Bag2 in HEK293T cells by co-immunoprecipitation. Plasmids, *Pink1-V5* and *myc-Bag2*, were cotransfected into HEK293T cells. For IP of myc-Bag2 (left panel), transfection of an empty vector and *Pink1-V5* was as one of controls. For reverse IP of Pink1-V5 (right panel), transfection of an empty vector and *myc-Bag2* was as one of control. B. Mitochondrial and cytosolic fractions from HEK293T cells transfected with *Pink1-Flag* and *myc-Bag2* were subjected to IP using myc antibody. The precipitated proteins were separated by SDS-PAGE for Western blots. C. The total cell lysate from HEK293T was used for IP of endogenous Pink1. The endogenous Bag2 was detected from IP of Pink1. * indicates the full length Pink1; # indicates the processed Pink1; > or < indicates the heavy chain of IgG.

Fig. 2. Bag2 regulates Pink1 stability. A. *Pink1-Flag* and *myc-Bag2* were coexpressed in HEK293T cells. Expression of Pink1-Flag or myc-Bag2 was observed from total cell lysates (left panel). *Pink1* was expressed in *Bag2* knock-down HEK293T cells. Overexpressed Pink1-Flag and myc-Bag2 (left panel) or endogenous Bag2 (right panel) were probed from total cell lysates. B. *Pink1- Flag* alone or with *HA-HSP90* or *myc-Bag2* was transfected into HEK293T cells. 24 hours after transfection, the total cell lysates were analyzed by Western blot analysis using indicated antibodies. C. Endogenous Pink1 was detected in HEK293T cells with expression of myc-Bag2 (left panel) or knockdown of Bag2 (right panel). * indicates the full length Pink1; # indicates the processed Pink1.

Fig. 3. Bag2 regulates Pink1 degradation in HEK293T cells. A. Ubiquitinylation of overexpressed Pink1 is inhibited by Bag2. *Pink1-Flag* or *Pink1(K219M)-Flag* along with *myc-Bag2* was coexpressed in HEK293T cells. Transfection of *Pink1-Flag* and an empty vector was used as a control. Where indicated, transfected cells were treated by MG132 for 2 hours. Flag tagged Pink1 or its mutant was IPed for ubiquitinylation analysis by Western blot (left and middle panels). In the left panel, the ubiquitinylation analysis was performed from HEK293T cells with expression of Pink1-Flag and overexpression of myc-Bag2. In the middle panel, the ubiquitinylation analysis was performed for detection of Pink1-Flag or Pink1(K219M)-Flag with expression of myc-Bag2. In the right panel, ubiquitinylation level of Pink1-Flag was examined with knockdown of Bag2. B. Expression or knockdown of Bag2 affects ubiquitinylation of endogenous Pink1. Ubiquitinylation of Pink1 was detected by IP of Pink1 from HEK293T cells that either expressed myc-Bag2 (left panel) or knockdown of Bag2 (right panel). C. The level of CHIP affects Pink1 level through regulating ubiquitinylation of Pink1. *Pink1-Flag* was transfected into HEK293T cells along with overexpression of CHIP or knockdown of CHIP. (Top Panels) 24 hours after transfection, the total cell extracts were subjected to Western blots for Pink1-Flag. (Lower panels) 24 hours after transfection, cells were treated with MG132 for 2 hours for ubiquitinylation analyses. D. Bag2 has no effect on Parkin level. *Myc-Bag2* and *GFP-Parkin* were cotransfected into HEK293T cells. 24 hours after transfection, cell lysates were subjected to Western blot analysis. * indicates the full length Pink1; # indicates the processed Pink1.

Fig. 4. Bag2 regulates Parkin translocation via Pink1. A and B. Bag2 expression regulates Parkin translocation in HEK293T cells. HEK293T cells were cotransfected with empty vector and *GFP*, empty vector and *GFP-Parkin*, *myc-Bag2* and *GFP* or *myc-Bag2* and *GFP-Parkin* as indicated. A: representative images from transfected cells treated by CCCP for 1 hour. GFP/GFP-Parkin (green), myc-Bag2 (red), mitochondrial Tom 20 (cyan) and nuclei (blue). B: Cells were untreated or treated with CCCP as indicated. GFP colocalization with mitochondrial marker Tom20 was analyzed under fluorescence-microscopy. The percentage of GFP/mitochondria colocalization was ascertained as a ratio relative to total GFP positive cells. C and D. *GFP-Parkin* or *GFP* was expressed in HEK293T cell with control siRNA or *Bag2* siRNA. C: representative images from transfected cells as indicated treated with CCCP

for 1 hour. D: the top panel demonstrates that siRNA for *Bag2* significantly knocked down Bag2, resulting in decrease of Pink1 but no effect on GFP-Parkin. The bottom panel is quantification of mitochondrial Parkin colocalized with Tom 20 with and without CCCP treatment as indicated. Data are presented as mean ± SEM of at least three independent experiments. E and F. The mitochondrial transloction of Parkin was analyzed by Western blots after cellular fractionation. E: Parkin-GFP was expressed in HEK293T cells along with overexpression of myc-Bag2 or knockdown of Bag2 in the presence of CCCP. F: HEK293T cells were transfected with myc-Bag2 or siRNA for Bag2. After CCCP treatment and cellular fractionation, the level of Parkin was analyzed by Western blots. The GFP-Parkin or endogenous Parkin was normalized to mitochondrial marker complex 1 for relative levels of Parkin and then compared to siCon or vector controls.

Fig. 5. Parkin translocation is regulated by Bag2 via Pink1 in cortical neurons. Primary cortical neurons were transfected with plasmids as described in Fig.4A and B. A. representative images showing distribution of GFP or GFP-Parkin in wild type or Pink1 deficient neurons with CCCP treatment with and without Bag2 expression as indicated. GFP/GFP-Parkin (green), myc-Bag2 (red), mitochondrial Tom 20 (cyan) and nuclei (blue). B. the percentage of number of puncta in GFP positive neurons colocalized with Tom 20 to total number of GFP positive neurons was evaluated. Data are presented as mean ± SEM of at least three independent experiments.

Fig. 6. Bag2 protects neurons through Pink1 and Parkin against MPP^+ challenge. A. Expression or Knockdown of Bag2 in cortical neurons modulates neuronal viability. A plasmid expressed *GFP* or *myc-Bag2* was transfected into primary cortical neurons. The number of GFP positive viable neurons with intact nuclei was normalized to total number of GFP positive neurons after MPP^+ treatment (upper panel). In lower panel, the neurons were transfected with control scrambled siRNA, *Bag2* siRNA, *Pink1* siRNA, *Parkin* siRNA, siRNAs for *Pink1* and *Parkin*, siRNAs for *Bag2* and *Pink1* or siRNAs for *Bag2* and *Parkin*. Viability was evaluated by MTT assay. The knockdown of Bag2 was confirmed by Western blot analyses. B. Bag2 plays a protective role through Pink1/Parkin signaling pathway. *WT*, *Pink1*$^{-/-}$ or *Parkin*$^{-/-}$ neurons were transfected with empty vector or *myc-Bag2* plasmid. The neuronal viability was evaluated by normalizing GFP positive neurons with intact nuclei to total GFP positive neurons. Data are presented as mean ± SEM of at least three independent experiments.

Fig. 1

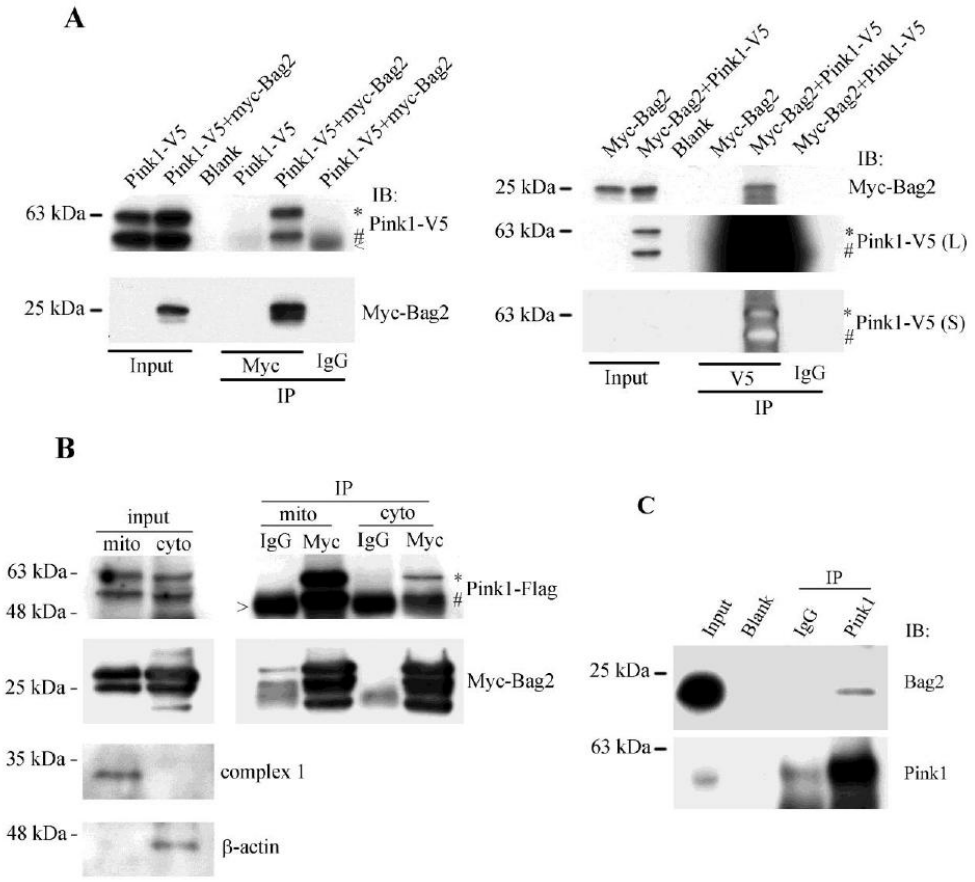
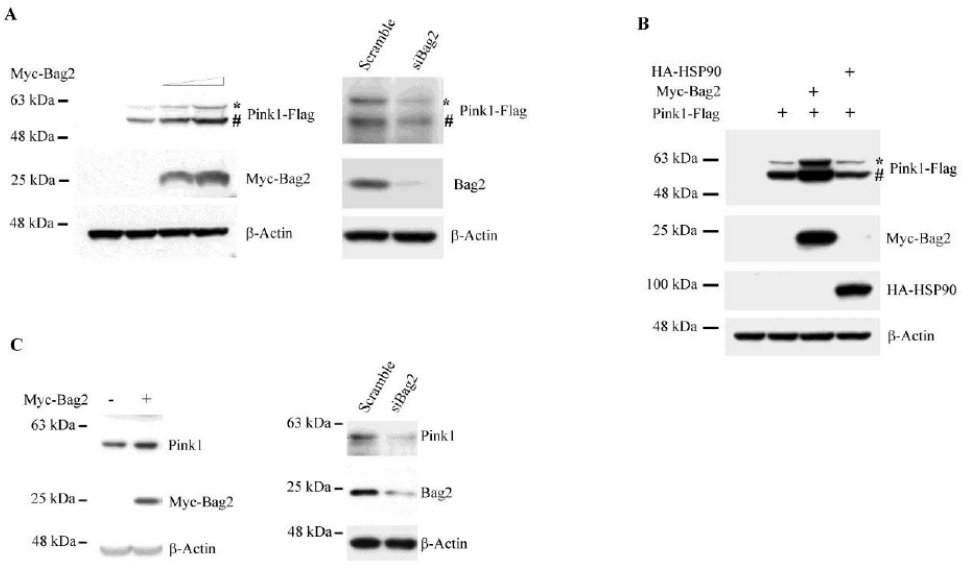


Fig. 2



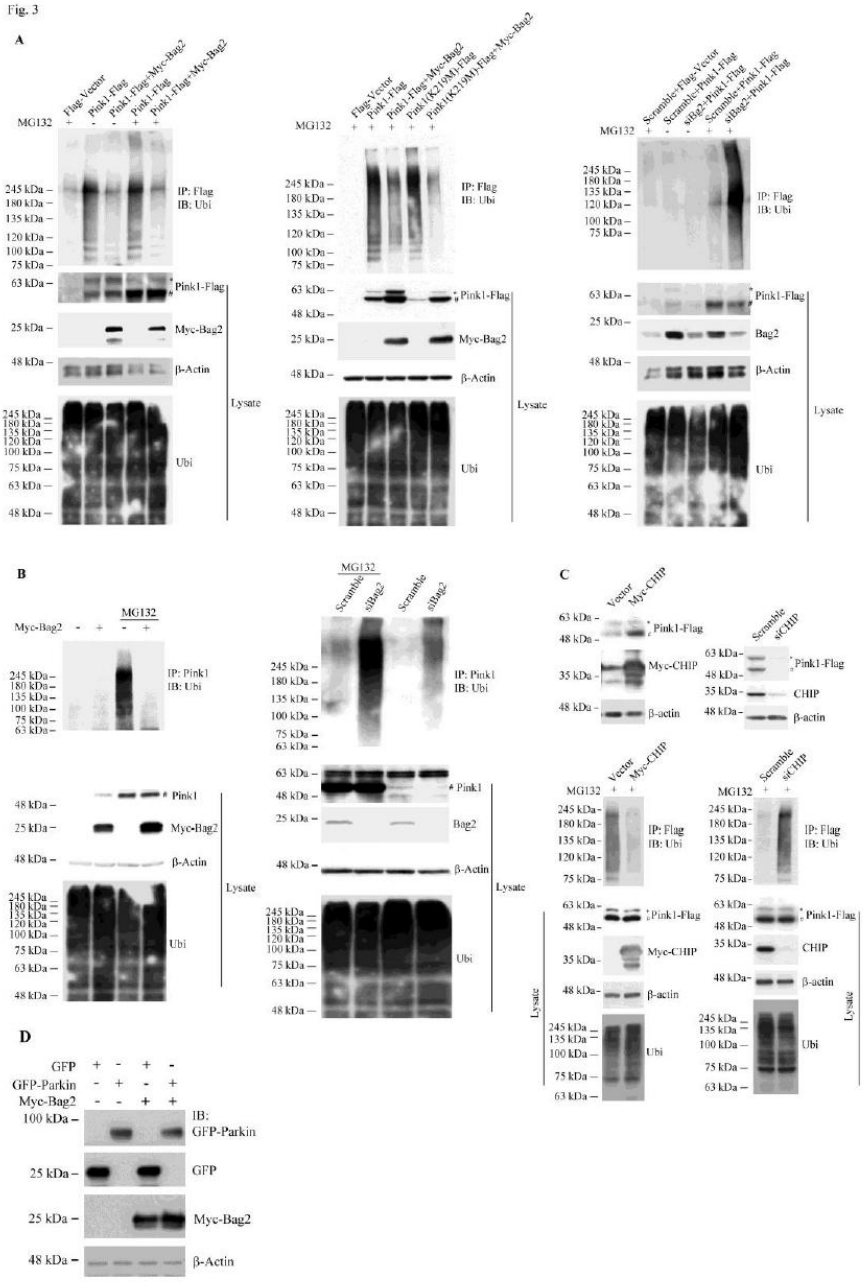


Fig. 4

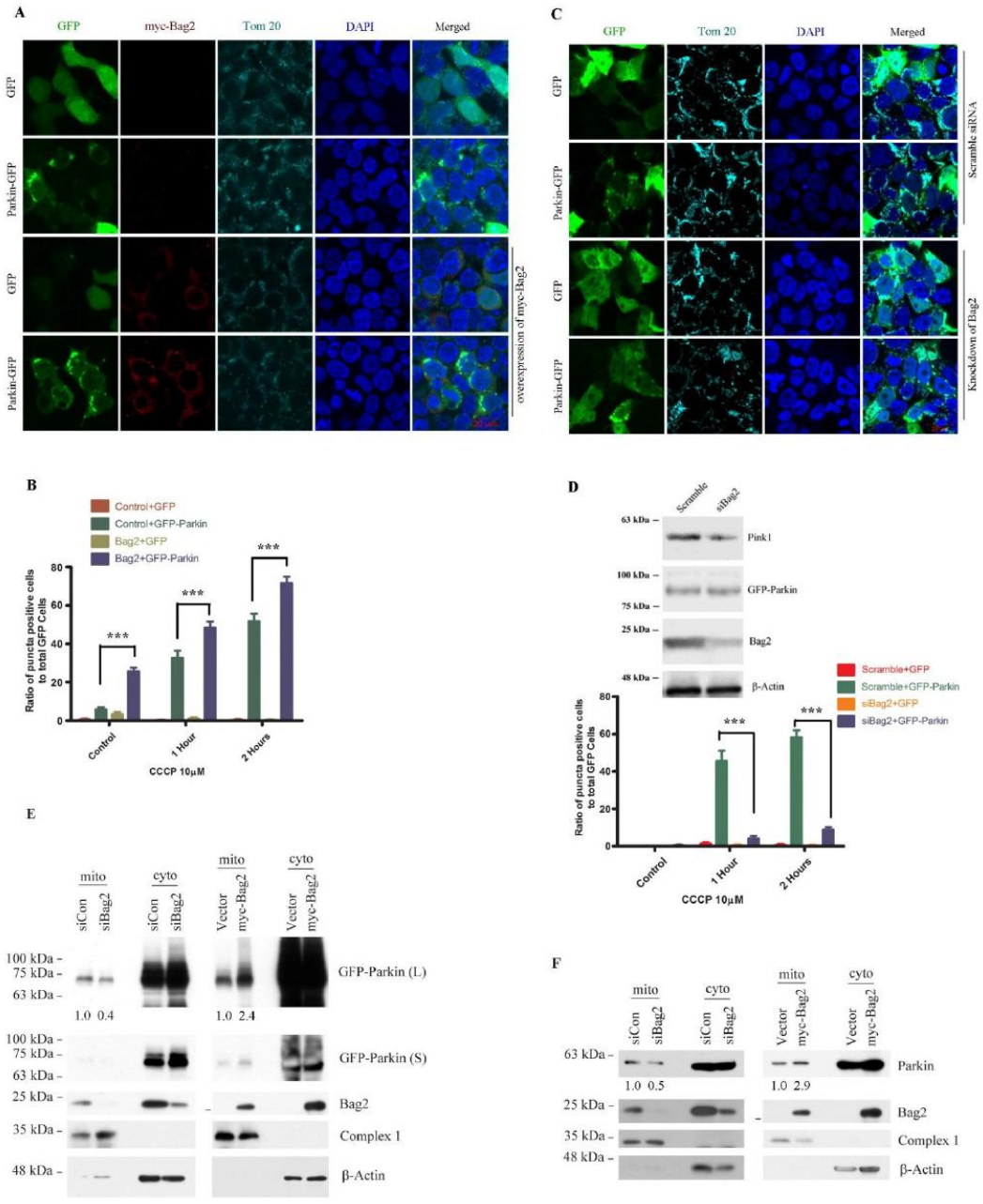


Figure 5

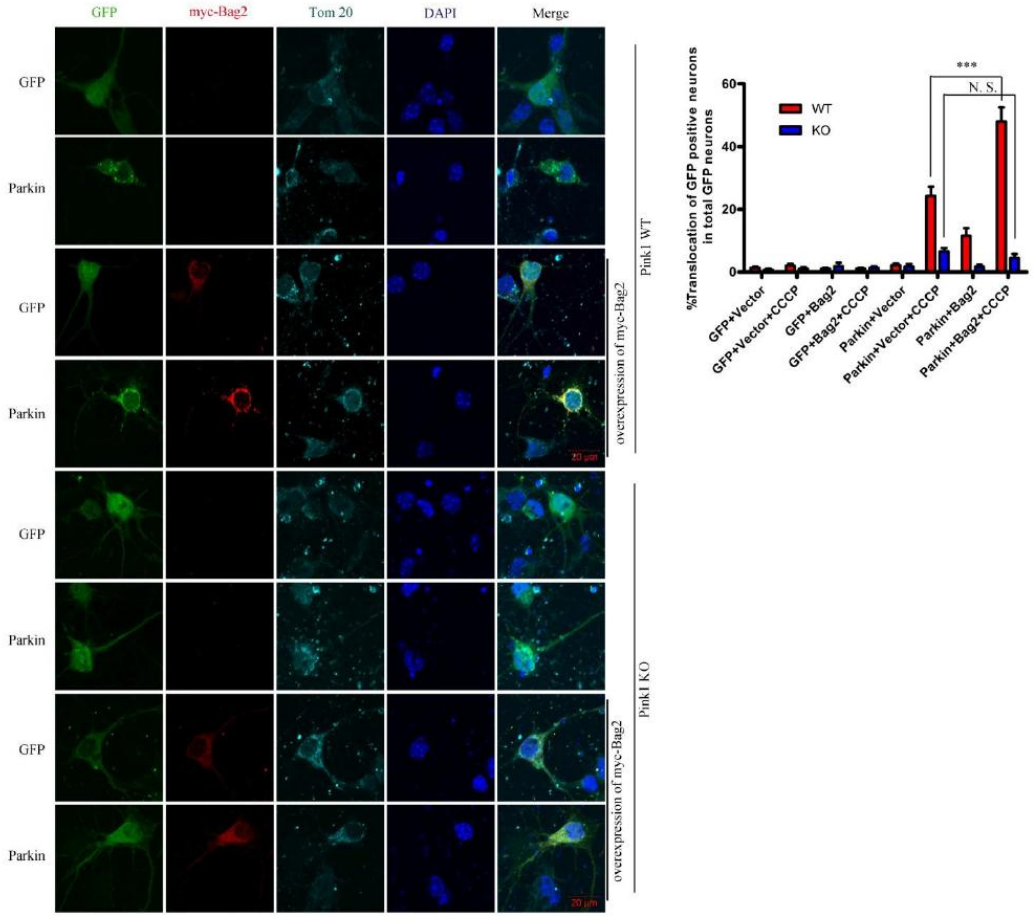
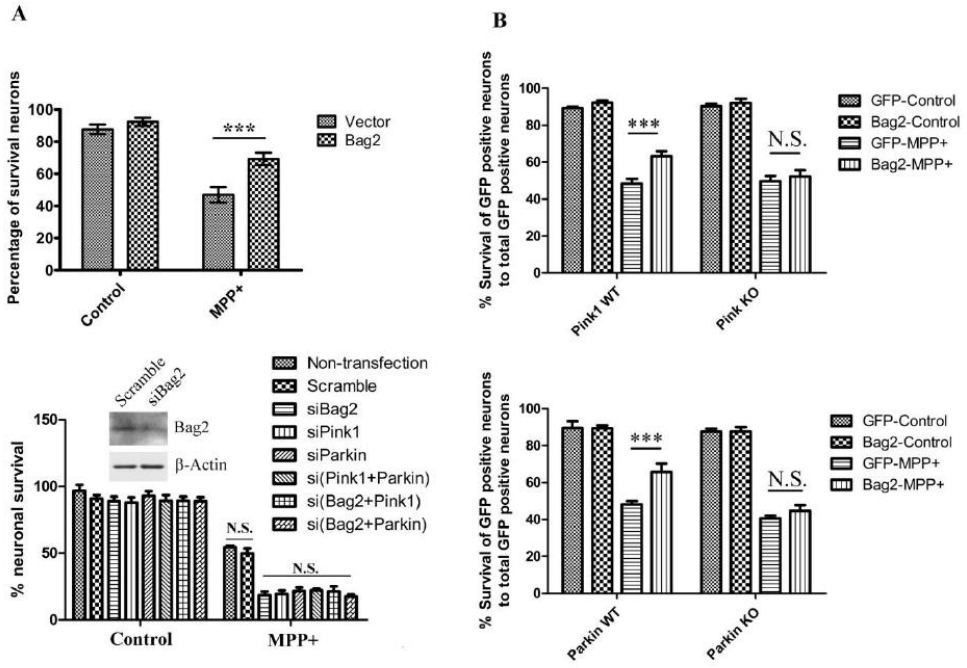


Fig. 6



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Contribution: My contribution to the research consisted of helping to perform some in vitro experiments including preparation of primary neuronal cultures. My overall contribution to this paper was 5%.

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Regulation of Ischemic Neuronal Death by E2F4-p130 Protein Complexes*

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Background: The contribution of E2F4 to hypoxic/ischemic neuronal death is understood poorly.
Results: Loss of E2F4 leads to an increase in B-Myb and contributes to hypoxic/ischemic neuronal death.
Conclusion: E2F4 is important for survival following hypoxic/ischemic neuronal death.
Significance: Targeting E2F4-repressive functions may be important in maintaining neuronal survival under hypoxic/ischemic conditions.

Inappropriate activation of cell cycle proteins, in particular cyclin D/Cdk4, is implicated in neuronal death induced by various pathologic stresses, including DNA damage and ischemia. Key targets of Cdk4 in proliferating cells include members of the E2F transcription factors, which mediate the expression of cell cycle proteins as well as death-inducing genes. However, the presence of multiple E2F family members complicates our understanding of their role in death. We focused on whether E2F4, an E2F member believed to exhibit crucial control over the maintenance of a differentiated state of neurons, may be critical in ischemic neuronal death. We observed that, in contrast to E2F1 and E2F3, which sensitize to death, E2F4 plays a crucial protective role in neuronal death evoked by DNA damage, hypoxia, and global ischemic insult both *in vitro* and *in vivo*. E2F4 occupies promoter regions of proapoptotic factors, such as B-Myb, under basal conditions. Following stress exposure, E2F4-p130 complexes are lost rapidly along with the presence of E2F4 at E2F-containing B-Myb promoter sites. In contrast, the presence of E2F1 at B-Myb sites increases with stress. Furthermore, B-Myb and C-Myb expression increases with ischemic insult. Taken together, we propose a model by which E2F4 plays a protective role in neurons from ischemic insult by forming repressive complexes that prevent prodeath factors such as Myb from being expressed.

Inappropriate activation of the cell cycle machinery is implicated in a number of neuronal death models induced by NGF deprivation (1), excitotoxicity (2), oxidative stress (3), DNA damage (4), and stroke (5–8). For example, an increase in cyclin D1 levels and its associated kinase activity is observed following DNA damage (an upstream mediator of stroke damage) and ischemic insult (5, 7, 9–11). In addition, treatment with pharmacologic cyclin-dependent kinase (Cdk)[3] inhibitors such as flavopiridol protects neurons from both DNA damage- and ischemia-induced cell death (4, 6, 7). Expression of a dominant negative form of Cdk4, an important regulator of the G_1/S phase of the cell cycle, is protective following DNA damage and global cerebral ischemia (5, 9). Together, these studies reveal a crucial role for Cdk4 in neuronal death induced by DNA damage and ischemic insult. However, the downstream effectors of cell cycle reactivation in neurons under ischemic stress remain unclear. In this regard, members of the E2F transcription factors may play a pivotal role.

The E2Fs consist of eight related family members, generally classified as activators (E2F1, E2F2, and E2F3a) or repressors (E2F3b and E2F4-E2F8) on the basis of their ability to promote or repress gene transcription (12). The activity of E2Fs (E2F1-E2F5) is regulated by their association with the pocket proteins, which include retinoblastoma protein (pRb), p107, and p130 (12, 13). E2F association with pocket proteins can promote or repress the expression of its targets, which include genes involved in cell cycle progression, DNA damage, and apoptosis (12, 13). For example, in dividing cells, pRb association with E2F prevents the expression of genes required for DNA synthesis and cell cycle progression. However, phosphorylation of pRb by Cdk4/6 disrupts its association with E2F and results in the transactivation of its target genes. In addition to transactivation, E2F complexes can also repress gene function. For example, E2F4-p130 can recruit chromatin modification factors, such as histone deacetylases (HDACs), to promoters of target genes to form an active repression complex (13–16). In this scheme, phosphorylation of p130 by Cdks disrupts its associa-

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[3] The abbreviations used are: Cdk, cyclin-dependent kinase; AAV, adeno-associated virus; CGN, cerebellar granule neuron; 4VO, four-vessel occlusion; CA1, cornu ammonis 1.

tion with E2F4, resulting in the derepression of target genes. Thus, E2Fs can function as effectors of Cdk signaling via the transactivation or derepression of target genes.

The involvement of E2F and, in particular, E2F1 in the cell cycle-induced death of neurons under pathologic stress is suggested by a number of key observations. First, phosphorylation of pRb, a preferred partner for activating E2Fs such as E2F1, is observed in neuronal death models, including DNA damage and ischemia (6, 7, 17). Second, the expression of kinase-dead Cdk4 and treatment with flavopiridol attenuates pRb phosphor ylation following DNA damage and ischemia (5, 7, 17). Importantly, the expression of mutant pRB or dominant negative DP-1, a binding partner of E2Fs, prevents neuronal death following DNA damage and/or hypoxia (5, 17). Finally, E2F1 deficiency is protective following ischemic insult both *in vitro* and *in vivo* (8, 18, 19). These observations suggest that cell cycle reactivation in neurons under pathologic stress signals death through pRb inactivation and activation or derepression of E2F1 target genes.

In addition to E2F1 and pRb, other E2Fs may also be important in neuronal death. E2F4-p130 complexes are present basally in postmitotic neurons, suggesting that they may act to repress gene targets important in neuronal survival/function (16, 20). Interestingly, in the absence of insult, siRNA-mediated down-regulation of p130 or E2F4 induced apoptosis of cortical neurons (21). In addition, the expression of wild-type or phosphorylation-resistant p130 is protective following NGF deprivation of PC12 neurons (21). Whether p130-E2F4 complexes play a role in neuronal death *in vivo* is unclear. In this study we examined the potential involvement of E2F4 in neuronal death induced by ischemic stress and DNA damage. The results of our study demonstrate an important role for E2F4 in the survival of neurons, which is in contrast to the role of activating E2Fs such as E2F1/3.

EXPERIMENTAL PROCEDURES

Viral Construction—Plasmids containing a human E2F4 cDNA sequence were subcloned into the SpeI sites of the AM/CBA-pI-WPRE-bGH vector and used to generate a recombinant adeno-associated (AAV) virus, as described previously (22). Plasmids containing Bmyb-luciferase with a wild-type E2F site and a mutant construct harboring a mutation that abolishes E2F binding have been reported previously (23). The plasmids were subcloned into the adeno-associated vector for efficient delivery into primary neurons. The adenovirus expressing E2F1 has already been described (24).

Transgenic Mice—E2F1- (25, 26), E2F3- (25, 27), and E2F4-deficient (28) mice have been described previously. Knockout mice were generated from heterozygous breeding pairs and genotyped by PCR using primers that have been published previously (26, 28). E2F1 null mice were genotyped using the following primers: 5′-GGATATGATTCTTGGACTTCTTGG-3′, 5′-CTAAATCTGACCCCAAACGC-3′, and 5′-CAAGT-GCCAGCGGGGCTGCTAAAG-3′.

Cell Cultures and Treatments—Primary cerebellar granule neurons (CGNs) and cortical neuronal cultures were established as described previously (24, 29) from CD1 (Charles River Laboratories, Quebec, Canada) or E2F transgenic mice. CGNs were transfected with E2F4 siRNA, a C-Myb siRNA mixture, or control siRNA (Santa Cruz Biotechnology) using Lipofectamine 2000 (30) 5 days after plating. Alternatively, CGNs were infected with an adenovirus expressing E2F1, E2F4, or GFP at the time of plating at a multiplicity of infection of 50. Cortical neuronal cultures were cotransfected with E2F1, E2F3, or E2F4 along with GFP-containing plasmids using the calcium phosphate method 3 days after plating, as described previously (30, 31). Cortical neurons were treated with 10 μM camptothecin (Sigma) 3–4 days after plating and examined for survival at the indicated times, as described previously (30). Cortical cultures from transgenic mice and CGNs were lysed at the designated times after insult using a mildly disruptive lysis buffer and evaluated as described previously (5). Neurons cotransfected with E2F and GFP plasmids were fixed and stained with Hoechst 33342 (Sigma). Viability was assessed by nuclear integrity in GFP-positive cells in random fields, as described previously (29).

Hypoxia—CGN cultures were subjected to hypoxia at 1% O_2 with 5% CO_2-balanced N_2 in a humidified hypoxia chamber (Coy Laboratory Products, Ann Arbor, MI) after 1 week in culture, as described previously (5). Hypoxia was induced in the presence of 10 μM MK801 (Research Biochemicals, Natick, MA), an NMDA channel blocker, for 18 h, followed by reoxygenation at normoxia for 24 h for survival studies. Alternatively, CGN cultures were subjected to varying durations of hypoxia and reoxygenation for biochemical studies. Control cultures were maintained in a humidified incubator at 37 °C and were not treated with hypoxia.

Viral Injection—Animal experiments were carried out in accordance with Canadian Council for the Use and Care of Animals in Research guidelines with approval from the University of Ottawa Animal Care Committee. Intrahippocampal, recombinant, adeno-associated viral injections in rats have been described previously (5). Briefly, male Wistar rats (80–125 g) were injected unilaterally with the recombinant adeno-associated virus carrying an enhanced green fluorescent protein (EGFP) control or E2F4 (10^{10} genomes/μl) 2 weeks before a four-vessel occlusion insult. 2 μl of recombinant adeno-associated virus diluted in PBS with 1 μl of mannitol (20%) was injected stereotaxically using a Harvard infusion pump (Harvard Apparatus) into the hippocampus (from the bregma, −3.6 mm anteroposterior, +2.1 mm lateral, and −2.75 mm deep).

Global Cerebral Ischemia—The four-vessel occlusion (4VO) method of global ischemia was induced as described previously for 10 min (6, 32). Four days following ischemia, rats were perfused and sacrificed, and the brains were extracted, sectioned, and stained for histological assessment. The neuronal viability of cells in the hippocampal CA1 was assessed as described previously (6, 32).

Immunohistochemistry—Antigen retrieval and deparaffinization was carried out on brain sections as described previously (32). Following permeabilization with 0.3% Triton X-100 for 10 min, rat brain sections were blocked in 10% normal goat serum (Jackson ImmunoResearch Laboratories) diluted in 2% bovine serum albumin (Fisher Scientific) in 0.01 M PBS for 1 h at room temperature. Sections were incubated with mouse mono-

clonal anti-GFP (Abcam) or anti-E2F4 antibodies (Abcam, 1:200) overnight at 4 °C. For visualization, sections were incubated with Alexa Fluor 488 goat anti-mouse (1:200) secondary antibody (Jackson ImmunoResearch Laboratories) for 1 h at room temperature. Neuronal nuclei were stained with Hoechst 33342 (Sigma).

Immunoprecipitation—CGN cultures were harvested and homogenized in immunoprecipitation buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, and 0.1% Tween 20) supplemented with protease inhibitor mixture (Roche). 500 μg of total protein lysate was incubated with 4 μg of anti-E2F4 (Santa Cruz Biotechnology) or anti-p130 antibody (Santa Cruz Biotechnology) overnight at 4 °C. As a control, samples were incubated with normal rabbit IgG (Santa Cruz Biotechnology). The antigen-antibody complex was captured with anti-rabbit Ig immunoprecipitation beads (eBiosciences) at 4 °C for 4 h. The beads were recovered, and the immunoprecipitation product was resolved on a 10% SDS-polyacrylamide gel.

Western Blotting—Cell and hippocampal tissue samples were collected at the designated times after insult and homogenized in solubilization buffer (0.0625 M Tris, 2.5 mM EDTA, 2.5 mM EGTA, 10% glycerol, 2% SDS, 0.001% bromphenol blue, and 5% β-mercaptoethanol) (24). Alternatively, E2F4 null CGNs or siRNA-transfected cultures were harvested and homogenized in solubilization buffer. Samples were run on SDS-polyacrylamide gels and transferred onto a PVDF membrane (Millipore). Membranes were probed with the following antibodies: anti-E2F4 (Abcam), anti-p130 (BD Transduction Laboratories), anti-phospho-p130 (Santa Cruz Biotechnology), anti-E2F1 (Santa Cruz Biotechnology), anti-E2F3 (Santa Cruz Biotechnology), and anti β-actin (Sigma). Densitometry was performed on Western blots using ImageJ software and normalized to the loading control. The results were expressed as the fold change over sham-operated animals in the 4VO time course and over no hypoxia control for hypoxia/reoxygenation experiments.

Semiquantitative Reverse Transcription PCR—At the indicated times following 4VO, total RNA was extracted from hippocampal tissues using QIAcube (Qiagen) following the protocol of the manufacturer. Alternatively, total RNA was extracted from siRNA-treated CGN cultures. 100 ng of total RNA was used for cDNA synthesis and targeted gene amplification using a SuperScript One-Step RT-PCR kit (Invitrogen). cDNA synthesis and amplification were conducted using the following conditions: 42 °C for 45 min, 94 °C for 2 min, followed by cycles of 94 °C for 1 min, T_m for 30 s, and 72 °C for 1 min. The rat MYB genes were amplified with the following primers: 5′-GGCTG CCGTGGCTACTACTTCTAA-3′ and 5′-CGCGC CGTTTC-TTCTGTCG-3′ for B-Myb at a T_m of 59 °C for 35 cycles and 5′-ATGCCCTGGAAGTGAAC AAC-3′ and 5′-CAGCTTTT-GTAAGCGGGTTC-3′ for C-Myb at a T_m of 54 °C for 35 cycles. Expression of GAPDH mRNA was used as a standard for loading control. GAPDH was amplified using the following primers: 5′-ATCCGTTGTGGATCTGACATGC-3′ and 5′-TGTCATTGAGAGCAATGCCAGC-3′ at a T_m of 52 °C for 28 cycles. The mouse C-Myb genes were amplified using the following PCR primers: 5′-GAGAGGTGGCACAACCATTT-3′ and 5′-GGGAACGTGACTGGAGATGT-3′ at a T_m of 54 °C for 31 cycles. The expression of ribosomal S 12 mRNA was used as a standard for loading control. S 12 cDNA was amplified using the following primers: 5′-GGAAGGCA TAGCTGC-TGG-3′ and 5′-CCTCGATGAC ATCCTTGG-3′ at a T_m of 57 °C for 25 cycles. Densitometry was performed on results and normalized to loading control. The results were expressed as fold change over control.

ChIP Assay—CGNs treated with/without hypoxia and reoxygenation were subjected to a ChIP assay as described previously (33). The mouse B-Myb promoter region was amplified using the following primers: 5′-CCTCCTCCTTC-TCCTCCTTC-3′ and 5-CACTATACCCGTGCGCTTCT-3′. PCR products were resolved on an agarose gel. Densitometry was performed on results and expressed as fold change over no hypoxia control.

Luciferase Assay—One day after plating, CGNs were infected with wild-type or mutant luciferase viruses along with AAV-β-galactosidase as an internal control. After hypoxia/reoxygenation, cells were lysed in buffer provided in the Promega luciferase system (Promega). The luciferase assay was performed according to the instructions of the manufacturer. Relative luciferase activities were obtained by normalizing the luciferase activity against β-galactosidase activity. Results were presented as fold increase in reference to control values.

RESULTS

E2F Family Members Differentially Regulate Neuronal Death Mediated by Genotoxic Stress—Aberrant activation of neuronal cell cycle induced by pathologic stress such as DNA damage and ischemic insult is thought to contribute to cell death through regulation of E2F members (7, 17, 33). However, the relative contribution of different E2F members in neuronal death is not clear. To begin to examine the involvement of different E2F members, we focused initially on the effect of select activating E2Fs (E2F1 and E2F3) and the repressive E2F4 members in neuronal death. Primary cortical neurons null for these E2F members were treated with the DNA-damaging agent camptothecin and evaluated for survival. Neurons null for E2F1 (Fig. 1*A*) and E2F3 (Fig. 1*B*) were more resistant to DNA damage-induced death when compared with those from their littermate controls. E2F1$^{-/-}$ neurons were significantly more resistant to DNA-induced death at 8 h (59% survival, $p < 0.01$) and 12 h (23% survival, $p < 0.05$) compared with the wild-type control (43 and 13% survival at 8 and 12 h, respectively). Similarly, neuronal survival in the E2F3$^{-/-}$ neurons (59 and 27%) was greater than that observed for the wild type (43 and 20%, $p < 0.01$ and $p < 0.05$ at 8 and 12 h, respectively). In contrast, E2F4 deficiency resulted in sensitization to DNA damage-induced death at 12 h (21% survival) compared with the wild-type control (44%, $p < 0.01$, Fig. 1*C*).

We next tested the effect of expression of E2F1, E2F3, and E2F4 on neuronal death induced by DNA damage. As shown in Fig. 1, *D–F*, expression of E2F1 (Fig. 1*D*) and E2F3 (Fig. 1*E*) significantly ($p < 0.01$) reduced neuronal survival compared with neurons transfected with vector only. In contrast, overexpression of E2F4 (Fig. 1*F*) resulted in an increase in neuronal survival (46% in vector- and 65% in E2F4-expressing neurons, $p < 0.01$). Together, these results demonstrate a proapoptotic

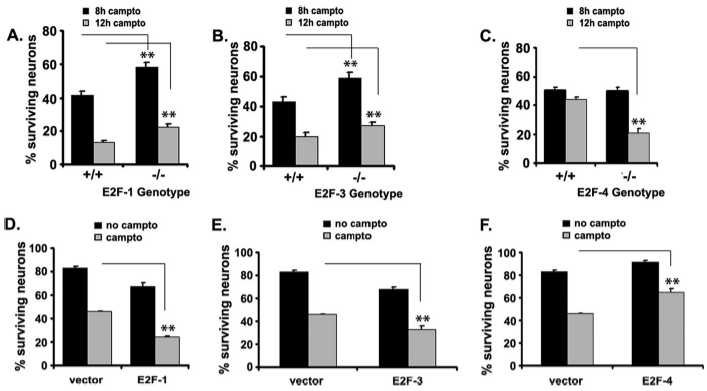

FIGURE 1. **E2F family members play differential roles in neuronal death induced by genotoxic stress.** E2F1 (*A*), E2F3 (*B*), and E2F4 (*C*) wild-type and knockout neurons were treated with 10 μM camptothecin (*campto*, a DNA-damaging agent) and evaluated for survival at 8 and 12 h following treatment. E2F1 (*D*), E2F3 (*E*), and E2F4 (*F*) containing a plasmid or vector control were transfected in cortical neuronal cultures 3 days after plating and treated with 10 μM camptothecin 24 h later. Survival was assessed 24 h following treatment. Results are expressed as percent of control ± S.E. **, $p < 0.01$.

role for E2F1 and E2F3 and a prosurvival role for E2F4 in neuronal death induced by DNA damage.

E2F1 and E2F4 Have Opposing Effects on Neuronal Survival following Hypoxia/Reoxygenation—We next determined the effect of E2Fs in a more physiologically relevant model of ischemic neuronal death *in vitro* with a focus on E2F1 and E2F4. To this end, primary CGNs infected with an adenovirus expressing E2F1, E2F4, or GFP were subjected to 18 h of hypoxia in the presence of MK801, followed by reoxygenation for 24 h. Neurons treated in this fashion die in a delayed manner dependent upon cell cycle activation (5). Consistent with our observation with DNA damage, the expression of E2F1 (Fig. 2*A*) significantly reduced neuronal survival compared with the GFP control (24% survival in E2F1- *versus* 45% in GFP-expressing neurons, $p < 0.01$). In contrast, expression of E2F4 was significantly protective following hypoxia/reoxygenation compared with the GFP control (65% survival in E2F4- *versus* 45% in GFP-expressing neurons, $p < 0.01$) (Fig. 2*A*).

Loss of function studies were consistent with the observations above (Fig. 2, *C* and *D*). In this regard, we focused on the role of E2F4 because loss of function studies with E2F1 following ischemic insult have been shown previously to promote survival (8, 18, 19, 34). CGNs were transfected with siRNA to E2F4 or control siRNA and subjected to hypoxia and reoxygenation. E2F4 knockdown in transfected cultures was verified by Western blot analysis (Fig. 2*B*). Evaluation of other E2Fs, including E2F1 and E2F3, showed that the levels of these proteins were not affected significantly by E2F4 siRNA-mediated knockdown.[4] We observed sensitization of neurons to death induced by hypoxia/reoxygenation when E2F4 was transiently knocked down (3% survival) compared with the siRNA control (29% survival, $p < 0.001$). Interestingly, neurons transfected with E2F4 siRNA were remarkably more vulnerable to neuronal death even in the absence of insult (19% survival in E2F4 knockdown *versus* 84% in siRNA control cultures, $p < 0.001$) (Fig. 2*C*). CGNs from E2F4$^{-/-}$ and E2F4$^{+/+}$ mice were also similarly subjected to hypoxia/reoxygenation. E2F4$^{-/-}$ neurons also showed an increased sensitivity to hypoxia-induced neuronal death (23% survival in E2F4 null *versus* 58% in wild-type neurons, $p < 0.01$) (Fig. 2*D*). These results suggest that, unlike E2F1, E2F4 plays a prosurvival role in neuronal death induced by hypoxia. Interestingly, the increased sensitization of E2F4 siRNA-treated neurons to death in the absence of insult suggests a potential compensatory effect in E2F4 knockout neurons. To examine this possibility, we analyzed E2F1 and E2F3 protein levels in E2F4 wild-type and knockout neurons by Western blot analysis. Our results showed that the E2F1 protein level is unaffected by the loss of E2F4 (Fig. 2, *E* and *F*). However, the E2F3 protein level was reduced significantly ($p < 0.05$) with germ line E2F4 loss (Fig. 2, *G* and *H*). This is in contrast with our earlier observation that E2F3 levels are unaffected by the transient knockdown of E2F4.[4]

E2F4 and p130 Proteins Are Reduced following Hypoxia/Reoxygenation in Vitro—E2F4 is known to form repressive complexes with p130 in neurons, which may be important in neuronal survival (21). Coimmunoprecipitation experiments showed that E2F4 and p130 normally form complexes in CGN (Fig. 3*A*). This is in accordance with previous reports (23, 35). We then asked whether there were perturbations in total E2F4 and p130 protein levels following hypoxia/reoxygenation insult. CGN cultures were subjected to varying durations of hypoxia or 18 h of hypoxia, followed by varying reoxygenation times. Total protein was harvested at the indicated times and analyzed by Western blot analysis (Fig. 3, *B* and *D*). The blots were probed with antibody against E2F4 (Fig. 3*B*) or p130 (Fig. 3*D*). E2F4 (Fig. 3, *B* and *C*) protein levels were diminished significantly ($p < 0.05$) following 18 h of hypoxia (*R0*) and remained reduced for up to 8 h following reoxygenation when compared with untreated cultures. Although there was a reinduction of E2F4 levels at 2 and 4 h of reoxygenation, it did not reach the levels of untreated controls. Similar to E2F4, p130 (Fig. 3, *D* and *E*) protein levels were also reduced drastically ($p < 0.05$) immediately following hypoxia, with a brief reinduc-

[4] G. O. Iyirhiaro, Y. Zhang, F. Safarpour, S. M. Callaghan, R. S. Slack, and D. S. Park, unpublished data.

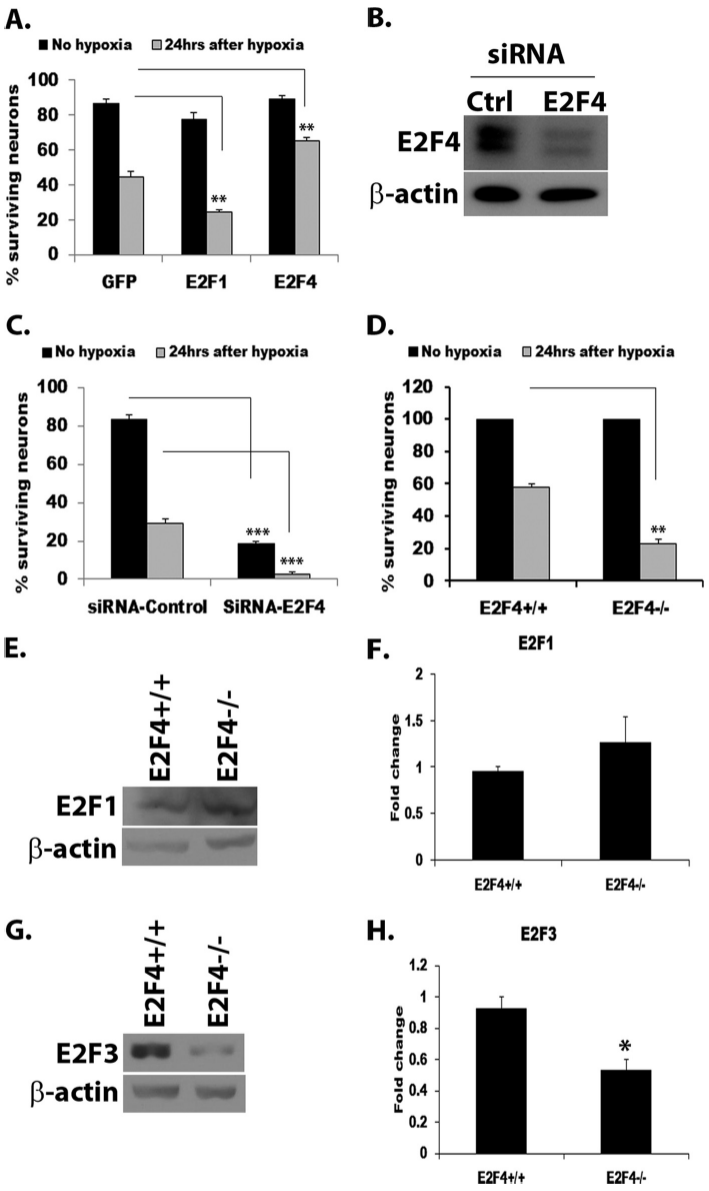

FIGURE 2. **E2F1 and E2F4 have opposing roles in neuronal death induced by hypoxia.** *A*, primary CGN cultures were infected with adenovirus expressing E2F1, E2F4, or GFP control at the time of plating and treated with hypoxia after a week in culture for 18 h, followed by reoxygenation for 24 h. *B*, Western blot analysis showing E2F4 knockdown in CGN cultures treated with E2F4 siRNA mixture. *Ctrl*, control. *C*, CGNs were transfected with E2F4 or control siRNA 5 days after plating. Cultures were treated with 18 h of hypoxia followed by 24 h of reoxygenation 2 days after transfection. *D*, CGNs from E2F4 KO and wild-type mice were similarly treated with 18 h of hypoxia and 24 h of reoxygenation. Neuronal survival was evaluated 24 h after hypoxia. Results are expressed as percent of control ± S.E. *E*, E2F1 protein levels are unaffected by E2F4 deficiency. CGNs from $E2F4^{+/+}$ and $E2F4^{-/-}$ neurons were subjected to Western blot analysis and probed with anti-E2F1 antibody and anti-β-actin as a control. *F*, quantitation of E2F1 levels as in *E*. *G*, E2F3 protein levels are diminished in E2F4 knockout neurons. A Western blot analysis was conducted on $E2F4^{+/+}$ and $E2F4^{-/-}$ CGNs. E2F3 was detected using anti-E2F3 antibody. β-actin was used as a loading control. *H*, quantitation of E2F3 protein levels as in *G* by densitometry. The protein levels of E2F1 (*E*) and E2F3 (*G*) were normalized to β-actin. *Error bars* represents the mean ± S.E. ($n \geq 3$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

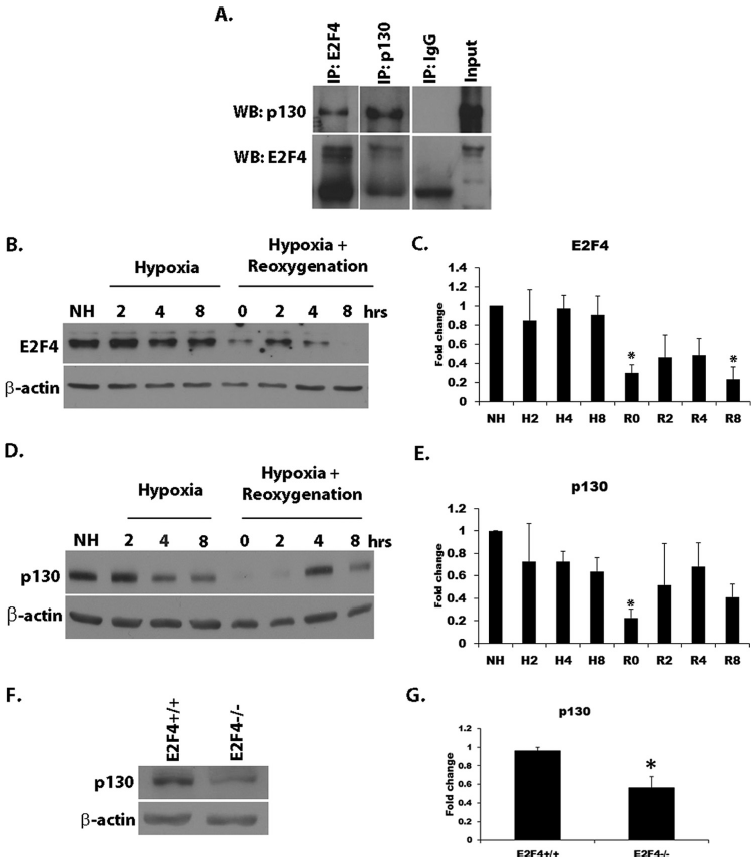

FIGURE 3. **p130 and E2F4 protein levels are down-regulated following hypoxia/reoxygenation of CGNs in culture.** *A*, E2F4 forms a complex with p130 in CGNs. Total cell lysates from CGN cultures were subjected to reciprocal immunoprecipitation (*IP*) with anti-E2F4 and anti-p130 antibodies. Immune complexes were resolved by Western blot (*WB*) analysis and probed with anti-E2F4 and anti-p130 antibodies. *B*, Western blot analysis showing a loss of E2F4 protein in CGNs subjected to varying durations of hypoxia or 18 h of hypoxia with varying durations of reoxygenation. *NH*, no hypoxia. *C*, densitometry was performed on E2F4 blot as in *B*. E2F4 protein levels were normalized to the actin control. *H*, hypoxia; *R*, reoxygenation; *R0*, 18 h of hypoxia and 0 h of reoxygenation. *D*, Western blot analysis showing the loss of p130 following different durations of hypoxia or 18 h of hypoxia with varying reoxygenation times. *E*, densitometry was performed on p130 protein levels as in *D* and normalized to the β-actin control. *F*, basal p130 protein levels were diminished in E2F4$^{-/-}$ CGNs. A Western blot analysis was conducted on total protein lysate obtained from E2F4$^{+/+}$ and E2F4$^{-/-}$ CGN cultures. The blots were probed with anti-p130 antibody and anti-β-actin as a control. *G*, densitometry was conducted on p130 blots as in *F*. p130 levels were normalized to β-actin. *Error bars* represent the mean ± S.E. ($n \geq 3$). *, $p < 0.05$.

tion at 2 and 4 h of reoxygenation. The reason for this induction is unclear at the moment. However, levels appeared to diminish again thereafter. The loss of p130 could be related to the loss of E2F4 following hypoxia and reoxygenation. Indeed, a Western blot analysis performed on E2F4$^{-/-}$ CGN cultures showed that p130 protein levels were diminished significantly ($p < 0.05$) in the absence of E2F4 (Fig. 3, *F* and *G*).

Hypoxia/Reoxygenation Induces a Concomitant Reduction of E2F4 and Induction of E2F1 Binding at the B-Myb Promoter—The previous evidence indicates that p130 is lost following ischemia. We next determined how E2F members such as E2F1 or E2F4 might differ in binding to known E2F sites. In this regard, we focused on B-Myb, a prodeath factor described previously for neurons and regulated by E2Fs (21, 35). We examined the relative promoter occupancy of both E2F1 and E2F4 following hypoxia/reoxygenation *in vitro*. ChIP was carried out on lysates from CGN cultures treated with 18 h of hypoxia and 16 h of reoxygenation using E2F1- and E2F4-specific antibodies. E2F1- and E2F4-associated chromatin was subjected to PCR using B-Myb promoter-specific primers. As shown in Fig. 4*A*, endogenous E2F4 occupancy at the B-Myb promoter was present in the untreated, no hypoxia samples under basal conditions. This occupancy decreased with hypoxia/reoxygenation (Fig. 4, *A* and *B*). This contrasts with E2F1, where occupancy is low under basal conditions and increases with hypoxic stress (Fig. 4, *A* and *C*). Consistent with the loss of E2F4, a ChIP analysis performed using p130-specific antibody also showed that it is lost from the B-Myb promoter following hypoxia/reoxygenation (Fig. 4, *D* and *E*). Interestingly, we

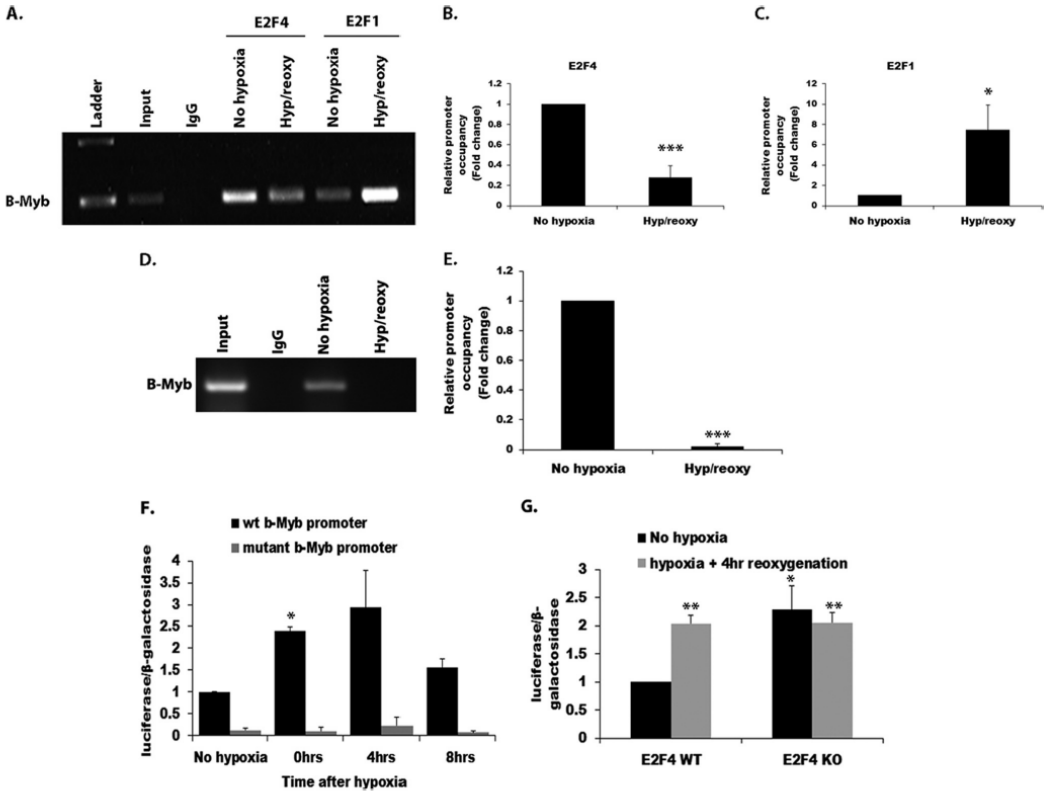

FIGURE 4. **Reduction of E2F4 and induction of E2F1 binding at the B-Myb promoter following hypoxia and reoxygenation.** *A*, ChIP was conducted with E2F4 and E2F1 antibody on CGNs treated with hypoxia (*hyp*) for 18 h, followed by reoxygenation (*reoxy*) for 16 h. *B*, densitometry of the B-Myb signal following ChIP performed with anti-E2F4 antibody as shown in *A*. Loss of E2F4 was observed at the B-Myb promoter following hypoxia and reoxygenation. *C*, quantitation of the B-Myb signal following ChIP with anti-E2F1 antibody. *D*, ChIP was performed with anti-p130 antibody. p130 binding at the B-Myb promoter was lost following hypoxia and reoxygenation. *E*, densitometry of the B-Myb signal following ChIP as shown in *D*. *F*, E2F activity increased after hypoxia and reoxygenation in CGNs. Cells were infected with AAV expressing B-Myb-promoter-luciferase with wild-type E2F or a mutated E2F site and β-galactosidase. Cells were treated with 18 h of hypoxia followed by reoxygenation for up to 8 h. Luciferase activity and β-galactosidase were measured at the indicated times. Data represent values of luciferase/β-galactosidase activity. *G*, a luciferase assay was conducted in E2F4 wild-type and knockout CGNs treated with 18 h of hypoxia followed by 4 h of reoxygenation. *Error bars* represent mean ± S.E. (n ≥ 3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

found that, in the absence of E2F4, p130 is lost from the B-Myb promoter under basal conditions.[4] This result indicates that E2F1 and E2F4 may mutually regulate B-Myb in an opposing manner to increase B-Myb expression and promote death. The ChIP experiments were performed at later time points where we could observe robust biochemical changes. To query the effects of E2F regulation at earlier time points, we next examined changes in E2F-mediated activity at the B-Myb promoter utilizing more sensitive, luciferase-based promoter assays. To this end, CGNs were infected with AAV expressing E2F reporter constructs, B-Myb-promoter-luciferase containing wild-type or mutated E2F sites as a control, and β-galactosidase 1 day after plating. The cells were treated with hypoxia and varying duration of reoxygenation after a week in culture. Luciferase and β-galactosidase analyses showed that E2F activity at the B-Myb promoter was induced significantly (2.5-fold, $p < 0.05$) immediately following hypoxia and remained elevated during reoxygenation (Fig. 4*F*). Because E2F4 is known to form repressive complexes and our results indicated that it is lost following hypoxic stress, we next investigated the effects of its deficiency on overall E2F activity at the B-Myb promoter. E2F4 wild-type and knockout CGNs were similarly infected with AAV expressing the same E2F reporter constructs as above, and luciferase activity was measured following 18 h of hypoxia and 4 h of reoxygenation (Fig. 4*G*). Consistent with our earlier results, luciferase activity increased 2-fold ($p < 0.01$) following hypoxia/reoxygenation in the E2F4 wild-type CGNs (Fig. 4*G*). In the E2F4 null CGNs, the basal luciferase activity was elevated (2-fold, $p < 0.05$) compared with E2F4 wild-type cultures and was not increased further with hypoxia/reoxygenation (Fig. 4*G*). This result indicates that E2F4 is important in the basal suppression of B-myb. It is important to note here that, because no death is observed basally (at least with non-stressed E2F4 knockout neurons), this suggests that multiple signals, including Myb, act in concert to promote death following ischemic insult.

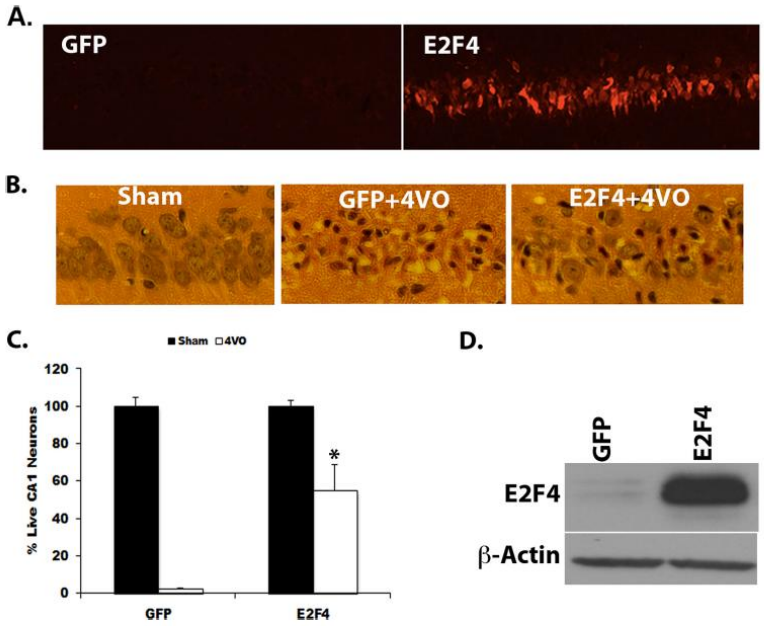

FIGURE 5. **E2F4 expression protects CA1 neurons from global cerebral ischemia.** *A*, immunofluorescence staining showing E2F4 overexpression in hippocampal CA1 neurons of rats injected with AAV expressing E2F4. *B*, hematoxylin and eosin-stained sections of CA1 neurons in sham- and 4VO-operated rats injected with GFP or E2F4 expressing AAV. *C*, quantitation of live CA1 neurons following 4VO in GFP and E2F4 injected rats. Rats were injected with AAV expressing E2F4 or a GFP control and subjected to sham surgery of 10 min of 4VO. Brains were extracted 4 days following reperfusion, sectioned, and stained with hematoxylin and eosin. H&E-stained coronal sections were evaluated for cell survival in the hippocampal CA1 ($n \geq 4$/group, data are mean ± S.E). *, $p < 0.05$ compared with GFP control. *D*, Western blot analysis of E2F4 expression in the hippocampus of GFP and E2F4 injected rats.

E2F4 Expression Is Protective following Transient Cerebral Ischemia in Vivo—To further investigate the role of E2F4 under ischemic conditions, we next examined its effects in an *in vivo* model of stroke induced by global ischemia. AAV expressing E2F4 or GFP control were injected unilaterally into the hippocampal CA1 region 2 weeks prior to insult, as described previously (5). Global ischemia was induced for 10 min using the four-vessel occlusion method, as described previously (6, 32). E2F4 overexpression and efficiency of viral delivery were verified by immunohistochemistry staining of CA1 neurons (Fig. 5*A*) and confirmed by Western blot analysis performed on hippocampal protein lysate from AAV-injected rats (Fig. 5*D*). Analysis of CA1 neurons 4 days following global ischemia showed significantly more live CA1 neurons (55% survival) in E2F4-expressing rats compared with those expressing GFP (3%, $p < 0.05$) (Fig. 5, *B* and *C*). GFP or E2F4 overexpression alone had no effect on neuronal viability in the absence of insult (Fig. 5*C*).

E2F4 and p130 Proteins Are Diminished following Global Ischemia—We also asked whether perturbations in E2F4 and p130 levels occurred *in vivo* following cerebral ischemia. Similar to the results obtained in CGNs, coimmunoprecipitation results showed that E2F4 and p130 also normally form complexes in rat hippocampal lysates.[4] To examine potential changes in E2F4 and p130 protein levels *in vivo* following ischemia, rats were subjected to 10 min of 4VO and sacrificed at various times following reperfusion, as described under "Experimental Procedures." Hippocampal lysates were extracted and subjected to Western blot analysis (Fig. 6*A*). The blots were probed with antibody against p130, E2F4, and actin for a loading control. p130 levels were increased (although not statistically significant) at early time points following ischemia but declined precipitously 24 h following reperfusion (Fig. 6, *A* and *B*). The early increase in p130 could be a compensatory response. Similarly to p130, the E2F4 protein level was reduced dramatically reduced 24 h following ischemia (Fig. 6, *A* and *C*). The biological function of p130 (14, 36) as well as its stability and down-regulation (37) are known to be regulated by Cdk-mediated phosphorylation. p130 has been shown to contain multiple Cdk phosphorylation sites, including Ser-952 (36). Therefore, we examined p130 Ser-952 phosphorylation following ischemia *in vivo*. Cdk-mediated phosphorylation of Ser-952 was increased following cerebral ischemia (Fig. 6, *D* and *E*). These results indicate that p130 is phosphorylated at early time points following ischemic insult and that both p130 and E2F4 proteins are subsequently lost in the death process. These results are similar to those observed with hypoxia/reoxygenation *in vitro*.

B- and C-myb Transcripts Are Induced following Global Ischemia in Vivo—Because our results showed that E2F activity is increased at the B-myb promoter *in vitro*, we examined potential changes in its transcript levels as well as that of the related C-Myb following ischemic insult *in vivo*. We observed that both B-Myb (Fig. 7, *A* and *B*) and C-Myb (Fig. 7, *C* and *D*)

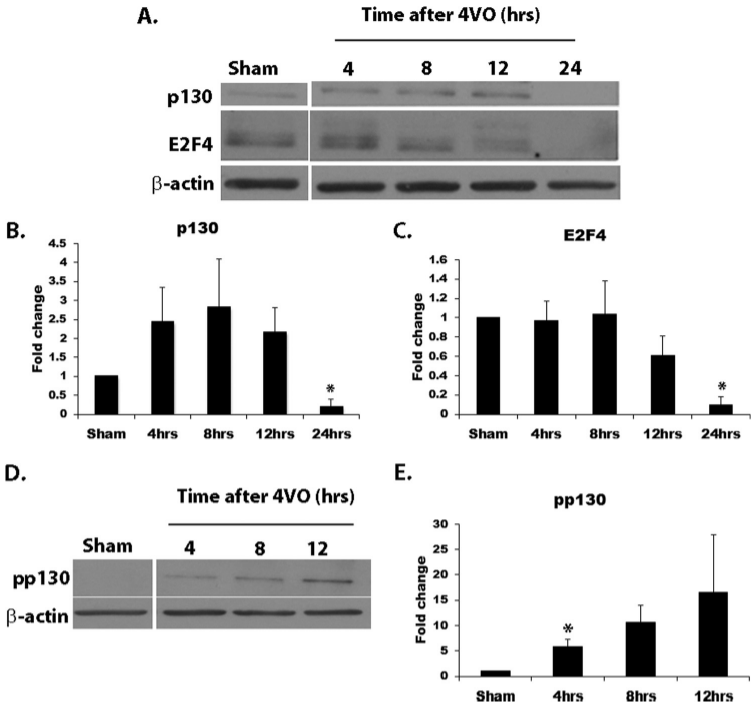

FIGURE 6. **p130 and E2F4 protein levels are decreased following global cerebral ischemia in rats.** *A*, Western blot analyses of p130 and E2F4 expression following 4VO in rats. Animals were subjected to 10 min of 4VO and sacrificed at the indicated times following reperfusion. Hippocampal tissues were extracted and subjected to Western blot analysis. Densitometry was performed on p130 protein levels (*B*) as well as E2F4 protein levels (*C*) and normalized to the actin control. *D*, Western blot analysis of p130 phosphorylation following 4VO. Rats were treated as in *A* and subjected to analysis using antibody directed at p130 phosphorylated at Ser-952. *E*, densitometry of Ser-952-phosphorylated p130. Expression levels were normalized to the actin control ($n \geq 3$). *, $p < 0.05$ compared with sham.

mRNA transcript levels were increased 3-fold ($p < 0.05$) 24 h following global ischemia. Overall, our results suggest that E2F4-p130 complexes provide neuronal protection under ischemic stress by regulating the expression of proapoptotic factors. Consistent with this hypothesis and previous reports (23, 35), we found that siRNA-mediated knockdown of the C-Myb gene provided protection for neurons subjected hypoxia/reoxygenation insult (Fig. 7*F*). CGN cultures were transfected with siRNA to C-Myb or control siRNA 5 days after plating and subjected to 18 h of hypoxia followed by 16 h of reoxygenation. Analysis of neuronal survival showed increased neuronal survival in neurons transfected with C-Myb siRNA (56%) compared with control siRNA (37%) (Fig. 7*F*). Knockdown of the C-Myb message was verified by semiquantitative RT-PCR in siRNA-treated cultures 24 h after transfection (Fig. 7*E*).

DISCUSSION

Cell cycle induced death of neurons has been described in numerous cell death paradigms (1, 20, 38–40). However, the downstream effectors of this pathway in the context of pathologic neuronal death are not fully understood, particularly in the context of ischemic damage. In this regard, a growing body of evidence suggests that death following ischemic insult is mediated via pRb-E2F1.

In line with this, we found that E2F1 deficiency was protective, whereas its overexpression exacerbated death induced by DNA damage and hypoxia/reoxygenation. Our data are consistent with a proapoptotic role demonstrated previously for E2F1 in other contexts, including death induced by staurosporine (41), β-amyloid (42), potassium deprivation (24, 43, 44), kainic acid (34), oxygen-glucose deprivation (OGD) (8), and ischemia (8, 24, 34, 41, 42). Similar to E2F1, E2f3, another member of the activating E2F family, is proapoptotic. Overexpression of E2F3 promoted cell death in response to camptothecin treatment, whereas its down-regulation was protective. This is in line with a previous report demonstrating a protective role for E2F3 deficiency in the developing CNS in response to DNA damage (45).

The role of the repressive E2F4 contrasts that of activating E2F members by promoting survival under conditions of ischemic stress. This is supported by several findings in this study. Acute down-regulation of E2F4 in CGNs using siRNA caused death in the absence of insult. Hypoxia/reoxygenation resulted in death that was exacerbated with E2F4 deficiency and knock-

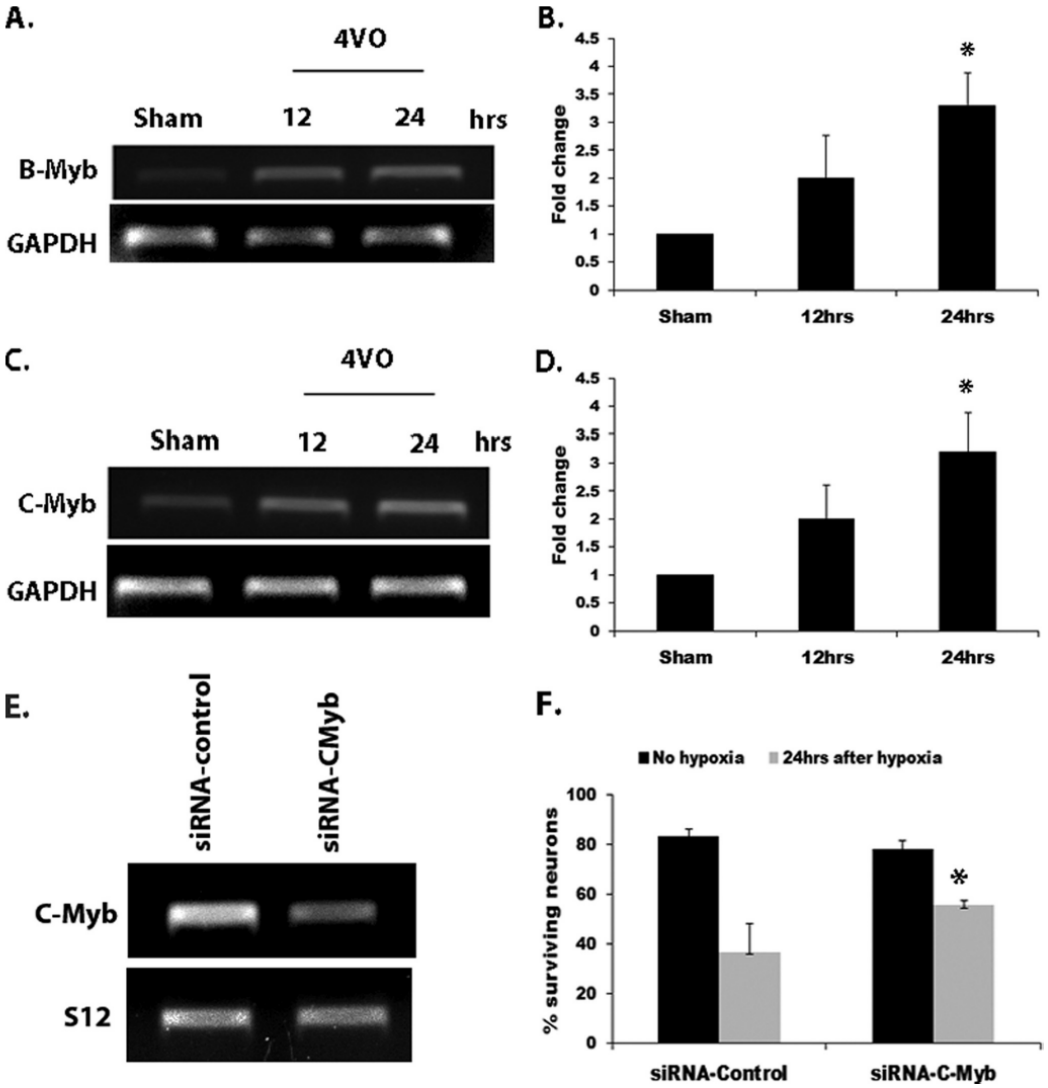

FIGURE 7. **B- and C-Myb mRNA transcript levels are increased following global cerebral ischemia.** *A*, semiquantitative RT-PCR of the B-Myb message following global ischemia. Rats were subjected to 10 min of 4VO followed by 12 and 24 h of reperfusion. Hippocampal tissues were extracted and analyzed by semiquantitative RT-PCR. GAPDH is shown as a control. *B*, densitometry of B-Myb levels. Data are expressed as fold over sham-operated control. *C*, semiquantitative RT-PCR of the C-Myb message following 4VO as described in *A*. *E*, semiquantitative RT-PCR showing siRNA mediated knockdown of the C-Myb message 24 h following transfection. S 12 is shown as a loading control. *F*, C-Myb knockdown protects CGNs from hypoxia/reoxygenation insult. Neurons were transfected with C-Myb siRNA and subjected to 18 h of hypoxia followed by 16 h of reoxygenation. *Error bars* represent the mean ± S.E. ($n \geq 3$). *, $p < 0.05$.

down in culture. In contrast, we found that overexpression of E2F4 protected neurons from death induced by hypoxia/reoxygenation *in vitro* and global cerebral ischemia *in vivo*. The data presented here as well as by others (8, 18, 19) demonstrate that, in the context of cerebral ischemic damage, the activities of both the activating and repressive E2Fs are an important determinant of neuronal survival and death.

The differential roles of activating and repressive E2Fs in neuronal survival is interesting. Although the exact reasoning behind this role is not clear, there are some intriguing hypotheses generated by our data. First, we showed that p130-E2F4 levels are present basally but reduced following ischemic insult. Second, we show that E2F4 binding to the E2F site on the B-Myb promoter is reduced following

hypoxia. This suggests a model where p130-E2F4 exists basally to form active repressive complexes but is lost after death-inducing insult. These repressive complexes may be critical for neuronal survival. In support of this, p130 predominantly occupies E2F sites in cultured neurons (21) and has been shown, along with E2F4, to participate in the mammalian DP, Rb-like, E2F and MuvB-like (DREAM) protein complex (46). Additionally, p130 levels are high in neurons (47, 48), and p130 loss has been suggested to promote death (49). In particular, p130 deficiency leads to strain-dependent lethality in null mice (49), and its down-regulation in cultured neurons promotes apoptosis (21).

In this scenario, we suggest that E2F4 is protective because it would facilitate the formation of repressive complexes, which are lost following ischemic insult. In contrast to E2F4, we propose that E2F1 acts to promote death by directly activating prodeath genes. This is supported in this study by the observation that E2F1 is prodeath and that its occupancy on death-inducing genes is increased following hypoxic insult. Therefore, we propose a model whereby hypoxia/ischemia leads to the loss of E2F4-repressive complexes on target promoters and allows for increase in E2F1 transactivating activity on those genes. Both would then contribute to an increase in expression of targets genes such as Myb, leading to death. Indeed, we show that targets such as B-Myb and C-Myb are increased following ischemia *in vivo*. It will be important to fully test this hypothesis in the future.

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Inactivation of *Pink1* Gene *in Vivo* Sensitizes Dopamine-producing Neurons to 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and Can Be Rescued by Autosomal Recessive Parkinson Disease Genes, *Parkin* or *DJ-1*

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Background: Mutations in *Pink1* are associated with Parkinson disease.
Results: Mouse *Pink1* deficiency results in hypersensitivity to MPTP-induced dopaminergic neuronal loss, which can be rescued with expression of human *Parkin* or *DJ-1*.
Conclusion: *Pink1* gene can regulate response to exogenous stress.
Significance: These results indicate how endogenous *Pink1* plays an important role in management of exogenous stress in mouse brain.

Mutations in the mitochondrial PTEN-induced kinase 1 (*Pink1*) gene have been linked to Parkinson disease (PD). Recent reports including our own indicated that ectopic *Pink1* expression is protective against toxic insult *in vitro*, suggesting a potential role for endogenous *Pink1* in mediating survival. However, the role of endogenous *Pink1* in survival, particularly *in vivo*, is unclear. To address this critical question, we examined whether down-regulation of *Pink1* affects dopaminergic neuron loss following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the adult mouse. Two model systems were utilized: virally delivered shRNA-mediated knockdown of *Pink1* and germ line-deficient mice. In both instances, loss of *Pink1* generated significant sensitivity to damage induced by systemic MPTP treatment. This sensitivity was associated with greater loss of dopaminergic neurons in the Substantia Nigra pars compacta and terminal dopamine fiber density in the striatum region. Importantly, we also show that viral mediated expression of two other recessive PD-linked familial genes, *DJ-1* and *Parkin*, can protect dopaminergic neurons even in the absence of *Pink1*. This evidence not only provides strong evidence for the role of endogenous *Pink1* in neuronal survival, but also supports a role of *DJ-1* and *Parkin* acting parallel or downstream of endogenous *Pink1* to mediate survival in a mammalian *in vivo* context.

Parkinson disease (PD)[3] is a neurodegenerative disorder characterized by loss of dopamine neurons and movement deficits (1). Several recessive genes (*Pink1*, *Parkin*, and *DJ-1*) have been linked with familial forms of the disease (2). How mutation of these genes leads to PD pathology is unknown. *Parkin* possesses an E3 ubiquitin ligase activity, and its loss is associated with mild mitochondrial defects (3). Expressed *Parkin* translocates to defective mitochondria in response to the loss of the mitochondrial membrane potential and mediates mitophagy (4–6). In contrast to *Parkin*, *DJ-1* has an atypical peoxyredoxin like peroxidase activity (7). Its expression or loss has been associated with cell survival, particularly in response to oxidative stress (8, 9).

Pink1, the third and most recent recessive PD gene identified, contains a mitochondrial targeting motif and a serine-threonine kinase domain (10). The physiological substrate(s) of *Pink1* are not fully defined. *Pink1* can phosphorylate two mitochondrial proteins, *Trap1* and *HtrA2* (11, 12). However, the physiological relevance of this phosphorylation needs clarifica-

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[3] The abbreviations used are: PD, Parkinson disease; AAV, adeno-associated virus; DA, dopamine; DAT, dopamine transporter; ISH, *in situ* hybridization; MPP^+, 1-methyl-4 phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MTN, medial terminal nucleus; *Pink1*, PTEN-induced kinase 1; SNc, Substantia Nigra pars compacta; TH, tyrosine hydroxylase.

tion. Growing evidence also suggests that *Pink1* is required for the proper maintenance and regulation of mitochondrial morphology and function (13–16). Germ line deletion of *Pink1* in *Drosophila* causes mitochondrial defects in indirect flight muscles and results in complete disruption of mitochondrial cristae (13, 14). However, this does not occur with *Pink1* germ line-deficient mice. Similarly, *Pink1* has been shown to be essential for the translocation of expressed *Parkin* to the mitochondria in response to mitochondrial depolarization (17). However, whether this pathway of mitochondrial quality control is critical for the pathology of PD is ultimately unknown.

At a more fundamental level, *Pink1* may be an important modulator of cell survival (18). *Pink1* deficiency by itself does not appear to induce neuronal loss (19, 20). However, whether *Pink1* is essential in response to exogenous stress is an intriguing possibility. Indeed, it has been shown that expression of *Pink1 in vitro* is protective in response to exogenous stress such as 1-methyl-4 phenylpyridinium (MPP^+), rotenone, and staurosporine (21–23). However, these studies suffer from the caveats of overexpression, and the role of endogenous *Pink1* in dopamine neurons *in vivo* is unknown. Here, we show that knockdown or knock-out of *Pink1* in the SNc area is more sensitive to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) insults. We also find that this sensitization is reversed by other PD-associated genes such as *Parkin* or *DJ-1* and that these genes can protect even in the absence of *Pink1*. This evidence suggests that there are functional interactions among these three recessive PD genes that have a demonstrable impact on environmental stress.

EXPERIMENTAL PROCEDURES

Mice—All procedures were approved by the University of Ottawa Animal Care Committee, and the animals were maintained in strict accordance with the Guidelines for the Use and Treatment of Animals put forth by the Animal Care Council of Canada and endorsed by the Canadian Institutes of Health Research. Germ line-deleted *Pink1* mice were a generous gift from Dr. J. Shen and were back-crossed to C57BL/6 for more than seven generations (19).

Generation of Mouse sh-Pink1 Adenovirus—The *Pink1* siRNA oligonucleotides (Ambion ID nos. 180640, 180641, and 180642) (directed to silence mouse *Pink1* as well as negative control siRNA (nontargeting siRNA) were purchased from Ambion and cloned into the pSilencer 3.0-H1 siRNA vector as reported previously (23). The shRNA fragment, including the H1 promoter, was subcloned to pAdTrack-CMV vector to generate adenovirus as previously described and validated (23). We used the siRNA (ID no. 180640) in the subsequent experiment because it showed the highest knockdown efficiency.

Generation of Human DJ-1 Adenovirus—We generated adenovirus-harboring human *DJ-1* as mentioned earlier (8). Briefly, adenovirus vector-expressing human *DJ-1* were generated by subcloning into pAdTrack-CMV vector (8) in which the expression of GFP and *DJ-1* is driven by two separate CMV promoters. Adenovirus was produced and titered as described previously (8).

Generation of Human Parkin Adeno-associated Virus (AAV)—GFP-*Parkin* AAV was a generous gift from Dr. Edward A. Fon (University of McGill, Canada). AAV was generated and purified as described previously (24).

Viral Gene Delivery in Vivo—Male, 8–10 week-old C57BL/6 mice were purchased from Charles River Laboratories. The mice were individually housed and were acclimated to the new environment before receiving the recombinant adenoviruses that expressed sh-*Pink1* or scrambled DNA as control. Adenoviruses (2 μl; 1×10^7 particles/μl per construct) were stereotaxically injected into the striatum (coordinates from bregma: anterior-posterior, +0.5 mm; medial-lateral, −2.2 mm; dorsal-ventral, −3.4 mm) at an infusion rate of 0.5 μl/min using a syringe pump (PHD2000; Harvard Apparatus). The reduction of *Pink1* message is as described before (23). In addition, we performed *in situ* hybridization (ISH) to show the down-regulation of *Pink1* in the virus-injected area especially in the SNc area as described below. Similarly, human *DJ-1* or GFP control adenoviruses were injected to the midbrain of *Pink1* KO and WT mice. The mice that received sh-*Pink1* or *DJ-1* were challenged with MPTP or saline as mentioned in the MPTP injection section.

AAVs harboring human *Parkin* or GFP control were diluted with 20% mannitol to obtain 1.3×10^5 virus particles/injection. The mannitol premixed viruses (2 μl) were stereotaxically injected into the SNc area (coordinates from bregma: anterior-posterior, −3.0 mm; medial-lateral, −1.6 mm; dorsal-ventral, −4.1 mm) at an infusion rate of 0.5 μl/min using a syringe pump as mentioned earlier. The mice were kept for 2 weeks for complete expression of *Parkin* or GFP, and then the animals were challenged with MPTP as mentioned below.

ISH of Mouse Pink1—ISH was performed as described previously (25) using digoxigenin-labeled antisense RNA riboprobes prepared by *in vitro* transcription from linearized plasmids containing partial sequence of the mouse *Pink1* gene. In brief, mouse *Pink1* cDNA was generated from mouse RNA by RT-PCR. The generated cDNA was used as a template to synthesize a 500-bp DNA fragment by PCR and cloned into pcDNA 3.1(+) vector in sense and antisense direction. Sense and antisense vectors were then used to generate digoxigenin-labeled RNA probes where sense is used as a negative control. Mouse brains were sectioned at 14-μm thickness and incubated with the above mentioned probe overnight and then processed for staining. The stained sections were analyzed on an Axioskop 2 microscope and images were captured using a Microfire camera. The ISH signal was assessed by densitometric analyses using ImageJ software (National Institutes of Health). Briefly, the average densities of striatal fields or individual neurons in the SNc area in both contralateral and ipsilateral sites were determined. Percentage signal was then calculated as ratio of the signal in the ipsilateral *versus* contralateral site and multiplied by 100.

MPTP Administration in Vivo—Mice were challenged with MPTP once a day for 5 consecutive days (25 mg/kg, intraperitoneal, measured as free base; MPTP-HCl; Sigma-Aldrich) 1 week after adenovirus injection to permit sufficient time for retrograde transport and expression of the adenoviral-derived proteins (26, 27). In the case of AAV,

MPTP injection started 2 weeks after initiation of viral injection. Mice used as control received an equivalent volume of saline (0.9%) once daily. Assessment of dopamine neuron survival was performed 2 weeks after the start of the MPTP dosing regimen.

Immunohistochemical Analysis of Tyrosine Hydroxylase (TH)-positive Neurons—Brain tissues from mice injected with MPTP or saline were collected for immunohistochemical analyses as described previously (26, 27). Antibodies used were TH (1:10,000; Immunostar), and immunoreactivity was visualized by using an avidin-biotin complex peroxidase reaction.

Assessment of Dopamine Neuron Loss in Vivo—For viral experiments, the loss of neurons in the SNc was determined by serial section analysis of the total number of TH neurons in the medial terminal nucleus (MTN) region. Intrastriatal administration of adenovirus results in the highest retrograde expression of the gene at this level (26). Briefly, mouse brains were collected and sectioned into 14-μm slices for TH staining. The total numbers of TH positive neurons in the MTN region (−3.08 to −3.28 mm of bregma) in the ipsilateral and contralateral hemispheres were counted separately from at least three sections for each animal. The average numbers of TH neurons in each site of the brain were calculated and presented in the graph. We counted the same subpopulations of TH neurons in the case of AAV injections because they were directly delivered in the MTN region. Cresyl violet staining and counting were similarly performed to validate the result of TH immunostaining as reported previously (26). Briefly, sections at the levels of the MTN as described above were assessed for healthy cells with the morphology and size of dopamine neurons. Quantitation is similar as described for TH counts.

For the evaluation of TH neurons of *Pink1* KO mice only, we employed optical fractionation (28) using Stereo Investigator (version 6; MicroBrightField, Williston, VT), as described previously (29). In brief, 40-μm brain sections were examined within the rostal and caudal limits of the SNc (−2.54 to −3.88 mm of bregma). For each brain, seven coronal sections were examined. After immunoblotting, mounting, defatting, and coverslipping, the thickness of the sections was measured with a *z*-axis microcreator according to the manufacturer's instructions. Sections were analyzed using a ×100 lens. Total number of TH-positive neurons was determined using the optical fractionator. Cresyl violet staining for *Pink1* KO mice was performed at one of the levels of the SNc area (−2.54 to −3.88 mm of bregma) where TH population is highest. The results are presented in number and percentage.

HPLC Analysis of MPP$^+$—Analysis of MPP^+ was carried as described previously (30). Briefly, *Pink1* KO and WT mice were injected with a single dose of MPTP (25 mg/kg, intraperitoneal, measured as free base; MPTP-HCl; Sigma-Aldrich). 90 min after MPTP injection, the mice were killed, and striata were collected and processed for HPLC analysis (31).

Statistical Analyses—Data analysis was carried out using independent two-tailed *t* test. Significance was marked by * when $p < 0.05$, ** when $p < 0.01$.

RESULTS

Dopamine Neurons of Pink1 Knock-out Mice Are Sensitive to DA Toxin MPTP in Vivo—We demonstrated previously that ectopic expression of WT *Pink1* in the SNc area protected the TH neurons against the dopaminergic toxin MPTP (23). The metabolite of MPTP, MPP^+, is transported to dopaminergic neurons by the dopamine transporter (DAT) where it blocks mitochondrial complex I function and results in degeneration (32). However, to demonstrate the role of *Pink1* in degeneration more definitely in the adult *in vivo* context, loss-of-function studies are required. Our first approach in this regard was to utilize *Pink1*-deficient mice. Recently, several groups generated germ line-deleted *Pink1* mice (19, 33). These *Pink1* KO mice do not show basal loss of DA neurons. However, subtle defects such as decrease in DA release have been observed (19). Accordingly, we initially challenged either WT or *Pink1*-deficient animals with a subchronic paradigm of MPTP as described under "Experimental Procedures" and examined dopaminergic neuron survival. We evaluated the entire SNc region (−2.54 to −3.88 mm of bregma) by optical fractionation/stereology for healthy TH-immunopositive neurons. Importantly, DA neurons of *Pink1*-deficient mice were more sensitive to MPTP than their WT littermates as shown in Fig. 1, *A* and *B*. We also performed cresyl violet staining at one of the level of SNc area as mentioned under "Experimental Procedures" to corroborate the loss of dopamine neurons further. We observed a trend similar to that obtained for TH immunohistochemical analysis (Fig. 1*B*). The TH-positive neurons of SNc project their processes to the striatum. These terminal fibers are enriched with the DAT. Therefore, we examined whether the loss of DA neurons in the SNc area correlated with terminal loss as evaluated by DAT staining. As expected, we found a significant loss of DAT staining in *Pink1*-deficient animals compared with littermate controls (54.76% striatal density in MPTP-treated WT animal *versus* 31.80% in *Pink1* KO animals when normalized with saline-treated WT animals) (Fig. 1, *D* and *E*). To ascertain that the greater loss of DA neurons or decrease in DAT density is not due to the increased production of MPP^+ from MPTP, we measured the MPP^+ in the striatum. Indeed, we found that equal amounts of MPP^+ available in both WT/KO mouse striatum (Fig. 1*F*).

shRNA-mediated Knockdown of Mouse Pink1 in SNc Area Sensitizes DA Neurons to MPTP—The use of germ line deficiencies brings up potential concerns of unforeseen confounds brought on by developmental compensation that may have little to do with the original function of the ablated gene. Because of these concerns, a more transient approach to *Pink1* knockdown was explored in conjunction with the deficient mice. We first generated shRNA viral vectors to *Pink1* which could be used in our *in vivo* adult MPTP model. We employed the shRNA sequence which we had previously shown to be effective in down-regulating mouse *Pink1* expression *in vitro* (23). We subsequently generated sh-*Pink1* adenovirus or a control shRNA virus (scrambled sequence with no known homology with mouse gene). The effectiveness of our sh-*Pink1* vector to silence *Pink1* in the SNc was evaluated utilizing ISH. This method was chosen due to the absence of reliable *Pink1* anti-

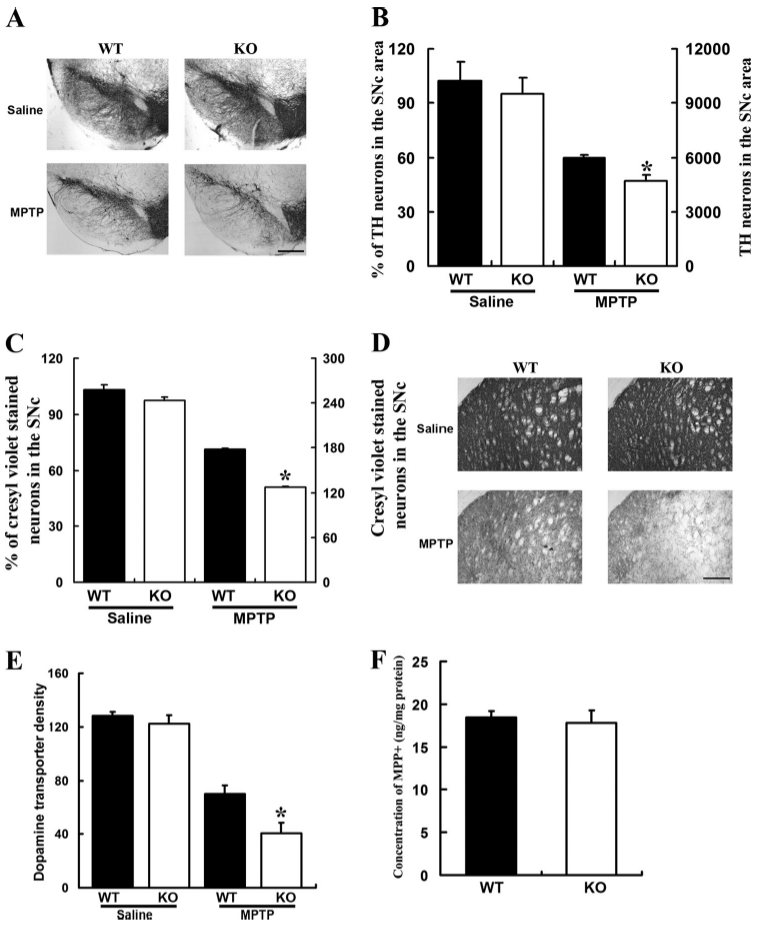

FIGURE 1. **Dopamine neurons of germ line-deficient *Pink1* mice are more sensitive to neurotoxin MPTP.** *A*, representative photomicrographs illustrating TH immunoreactivity in the ventral midbrain SNc. *Scale bar*, 250 μm. *B*, quantification of TH-immunoreactive neurons in SNc area of *Pink1* WT/KO animals by stereology, as described under "Experimental Procedures." *C*, cresyl violet-stained neurons in SNc area. *D*, representative photomicrographs illustrating DAT immunoreactivity in the striatum of *Pink1* WT/KO animals. *Scale bar*, 500 μm. *E*, quantification of optical density of striatal DAT-stained fiber density of *Pink1* WT/KO animals. *F*, HPLC analysis of striatal MPP^+ levels in *Pink1* WT/KO animals. Values are means ± S.E. (*error bars*; $n = 3–5$). *, $p < 0.05$ compared with WT animals treated with MPTP.

bodies needed for immunohistochemical analysis of endogenous *Pink1 in vivo*. The adenovirus was injected into the striatum area which retrogradely transports to the SNc. A week after the injection, the brain tissues were prepared for ISH to detect the *Pink1* signal. As shown in Fig. 2, *A–C*, the *Pink1* message signal was significantly lower (71%) in the striatum of the ipsilateral side, where viral injection was carried out, relative to the contralateral side. Most importantly, this reduction was clear in the area of the SNc in cells with the shape and size of DA neurons (68% reduction). One week after viral transduction with either sh-*Pink1* vector or control, animals were challenged with the same subchronic regimen of MPTP utilized with the *Pink1*-deficient animals as described earlier. Two weeks after the initial MPTP or saline dose, animals were killed, and the midbrain was sectioned. The number of TH-immunopositive neurons in both virus-injected (ipsilateral) and virus-uninjected (contralateral) in the SNc at the level of the MTN was assessed. Consistent with the germ line deficiency data, we found that silencing of *Pink1* in the SNc area led to increased loss of DA neurons in response to MPTP (Fig. 2, *D* and E). Silencing of *Pink1* by itself did not significantly affect TH neuron numbers, at least in the 3-week time frame examined. This result is in line with recent reports indicating that *Pink1* knockdown or knockout does not cause any DA neuron loss (19, 20, 33). To further ensure that the loss of TH neurons was not due to simple loss of TH marker expression and not degeneration, we also carried out cresyl violet staining and analyses of the SNc in adjacent sections at the level of the MTN. As shown in Fig. 2*F*, results similar to TH counts were obtained. Taken together, these results clearly indicate the critical role of endogenous *Pink1* in

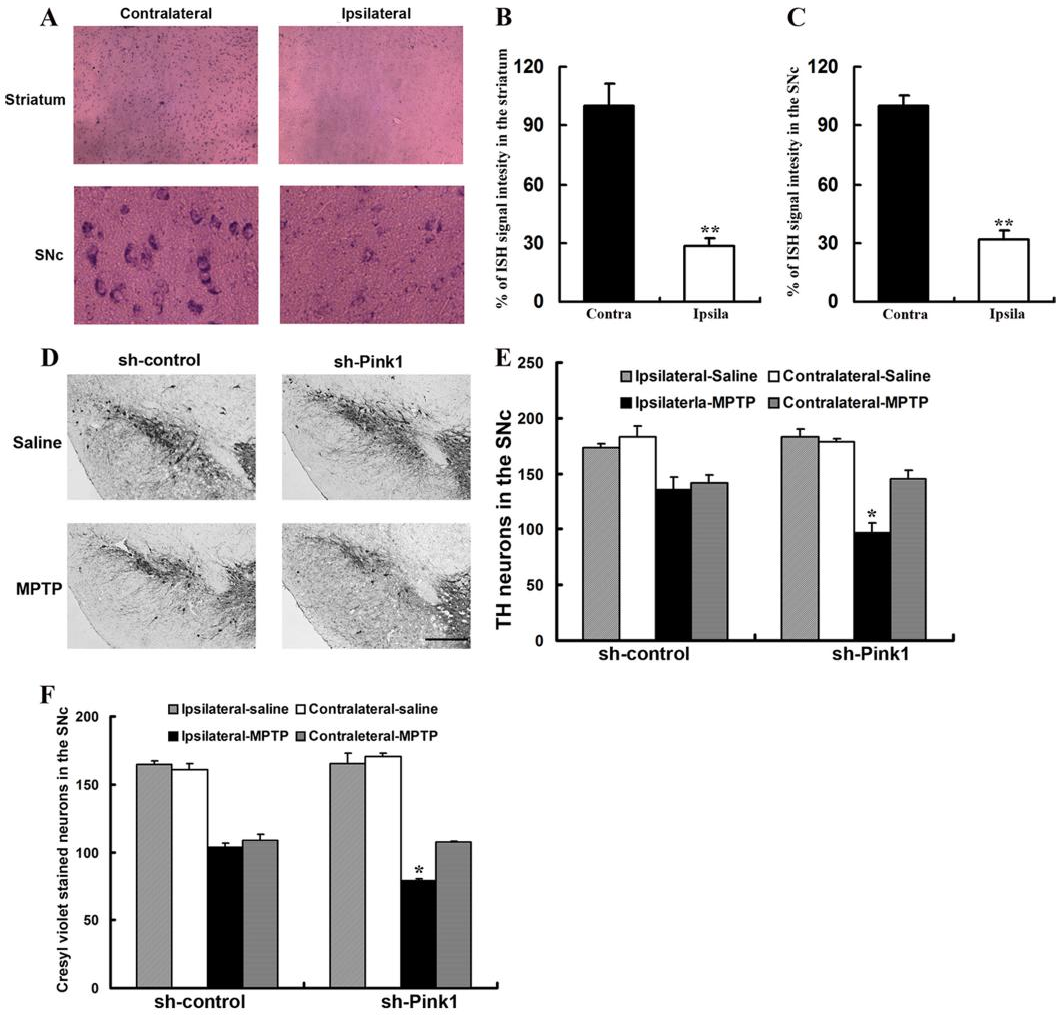

FIGURE 2. **shRNA-mediated knockdown of *Pink1* results in greater loss of dopamine neurons upon MPTP treatment.** *A*, adenoviruses (2 μl, 1×10^7 particles/μl) expressing *Pink1* shRNA or control injected directly into the striatum (ipsilateral side) of the animals. A week after viral injection, the brains were collected, fixed, and sectioned. The brain sections were processed for ISH to examine *Pink1* transcript in striatum and SNc area. *B* and *C*, percentage of ISH signal intensity in the striatum (*B*) and SNc (*C*). **, $p < 0.01$ *versus* contralateral site. *D*, adenovirus-injected mice challenged with MPTP as described under "Experimental Procedures." Brains were collected and sectioned into 14-μm slices for TH immunostaining. Representative images of TH-immunoreactive neurons of the ipsilateral side of the animals, 2 weeks after treatment with MPTP or saline, are shown. *Scale bar*, 250 μm. *E* and *F*, quantifications of TH-immunoreactive neurons from the ipsilateral or the contralateral region of SNc area (*E*) or cresyl violet-stained neurons (*F*). All values are means ± S.E. (*error bars*; $n = 3$–5). *, $p < 0.05$ compared with control virus-injected (ipsilateral) side of MPTP-treated animals.

neurons in mediating neuronal survival to exogenous stress in the adult animal *in vivo*.

Role of Parkin and DJ-1 in Modulating Sensitization Observed with Pink1 Loss—A functional relationship between *Pink1* and the other recessive PD genes, *Parkin* and *DJ-1*, has been suggested. For example, studies in *Drosophila* indicated that *Parkin* acts downstream of *Pink1* to rescue the abnormal wing and mitochondrial defects in indirect flight muscles, a phenotype associated with *Pink1* loss of function, at least in flies. Similarly, *Parkin* accumulates in damaged mitochondria and enhances mitophagy in a *Pink1*-dependent manner (4, 6, 17). However, in this case, the role of this translocation in mediating survival is unknown. Importantly, the capacity of *Parkin* to compensate for *Pink1* loss-mediated sensitization to death or to protect in the absence of *Pink1* is unknown. To test this, we examined whether *Parkin* expression in dopaminergic neurons can also rescue sensitization effects of MPTP due to absence of *Pink1*, *in vivo*. We delivered *Parkin*

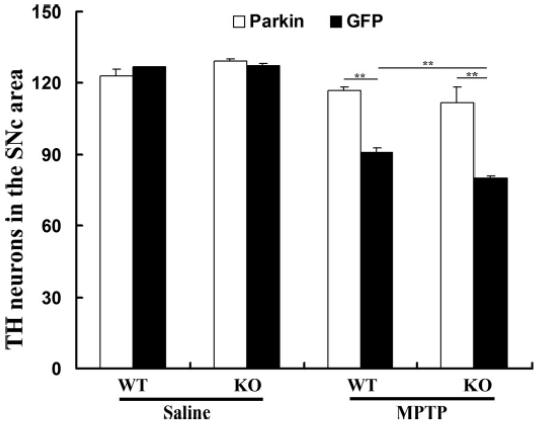

FIGURE 3. **Expression of AAV *Parkin* at the SNc area rescues DA neurons of *Pink1* KO mice from MPTP sensitization.** The AAVs expressing human *Parkin* or control were injected directly into the SNc area of animals 2 weeks before the initiation of MPTP treatment. Brains were collected and sectioned into 14-μm slices for TH immunostaining. Quantifications of TH-immunoreactive neurons from the ipsilateral region of SNc area of mice brain of different groups are shown. All values are means ± S.E. (*error bars*; $n = 3–4$), except GFP-injected saline-treated WT control ($n = 2$).

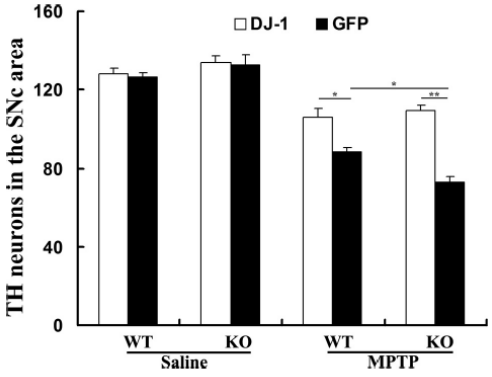

FIGURE 4. **Delivery of adenovirus-expressing *DJ-1* at striatum area rescues DA neurons of *Pink1* KO mice from MPTP sensitization.** The adenoviruses (2 μl, 1 × 10^7 particles/μl) expressing human *DJ-1* or control were injected directly into the striatum of animals 7 days before the initiation of MPTP treatment. Brains were sectioned into 14-μm slices for TH immunostaining. Quantifications of TH-immunoreactive neurons from the ipsilateral region of SNc area of mice brain of different groups are shown. Values are means ± S.E. (*error bars*; $n = 3–4$).

or GFP control AAV to the SNc 2 weeks prior to a challenge with MPTP. We found that expression of *Parkin* is effective in rescuing the sensitization induced by *Pink1* loss. It is important to note that in this case, the sensitization induced by *Pink1* deficiency was slightly lower (although still significant) than that observed in Fig. 1*B*. This may be due to the effects of viral transduction. Interestingly, *Parkin* provided the same extent of neuroprotection in the absence or presence of endogenous *Pink1* (Fig. 3). This result suggests that *Parkin* not only compensates for *Pink1* deficiency, but also provides further protection even in the absence of *Pink1*. This in agreement with the recent reports showing that ectopic expression of AAV *Parkin* was protective against MPTP under WT backgrounds (34, 35).

DJ-1 is an another PD gene involved in scavenging oxidative stress in multiple model systems (8, 9, 36). We have previously shown that loss of *DJ-1* sensitizes animals to multiple environmental stressors such as ischemia and MPTP *in vivo* (8, 9). Given its roles in processes such as mitochondrial function (37) and survival (9) which are also implicated for *Parkin* and *Pink1*, we next tested whether *DJ-1* might also compensate for *Pink1* loss. In this case, *DJ-1* or GFP controlled adenovirus was delivered stereotaxically to the striatum in either WT or KO *Pink1* animals. We have previously shown *DJ-1* to be effective in promoting survival *in vivo* with this virus (8). As shown previously, GFP-injected *Pink1* KO animals showed hypersensitization to death compared with WT controls in response to MPTP. Importantly, exogenous *DJ-1* expression was effective at overcoming this sensitization. Similar to *Parkin* expression, *DJ-1*-expressing animals showed the same level of survival at the SNc whether or not *Pink1* was present (Fig. 4). These results indicate that the other two recessive PD genes *Parkin* and *DJ-1* can protect even in the absence of *Pink1*. This suggests that they are either downstream of *Pink1* or on parallel pathways to regulate survival.

DISCUSSION

Recent discoveries of several genes associated with familial PD have given us the opportunity to enhance our molecular understanding of this devastating disease. Among these genes, *Pink1* has received much attention due to its clear mitochondrial targeting motif, its well defined kinase domain, and involvement in mitochondrial quality control pathways. Yet, the mechanism by which its loss leads to PD in humans is still unclear.

Role of Pink1 in Survival—One critical aspect of *Pink1* function has to do with its potential role in survival. As stated previously, it has been reported that WT *Pink1* expression blocks death against a number of death stimuli including MPP^+, rotenone, staurosporine and MG-132 (10, 21–23). We likewise observed this phenomenon in primary neurons expressing WT human *Pink1* (23). However, the endogenous role of *Pink1*, particularly in the adult mammalian brain *in vivo*, is less clear. This question is made more significant given recent questions on the relevance of seemingly critical biological processes such as *Parkin*-mediated mitochondrial quality control in the adult animal (38). This reflects a broader question of relevance of *in vitro* findings in the adult *in vivo* context.

Pink1 deficiency in mice does not lead to increased basal DA neuronal loss as observed in human PD. Our present data demonstrating that shRNA knockdown of *Pink1*, at least in the time frame examined, did not lead to increased basal DA loss is consistent with these results. In the fly, *Pink1* deficiency does lead to demonstrable DA loss, but the reason for this difference is unclear. One explanation may be that in the mammalian system, there are other survival pathways that are sufficient to prevent basal DA loss at least under basal conditions in the lifetime of the mouse. These compensatory pathways likely do

not involve *DJ-1* and *Parkin* because *Pink1*/*DJ-1*/*Parkin* triple KO mice also do not show basal DA loss (39).

Rather than focus on basal processes, the effects of exogenous environmental stress might better illuminate the functional role of *Pink1* in survival. Because of the role of *Pink1* in mitochondrial function/quality control and its localization, the effect of *Pink1* loss on mitochondrial stress is particularly interesting. This is even more critical given the increased association of mitochondrial defects in PD and the links of variety of PD genes to mitochondrial processes. Accordingly, we examined how *Pink1* loss may affect DA survival in response to the mitochondrial complex I toxin MPTP, to address the central question of whether endogenous *Pink1* plays a role in DA survival in the adult mammalian brain. Our results support the veracity of these hypotheses as follows: (i) germ line-deficient *Pink1* animals are more susceptible to MPTP and (ii) this is likely not due to developmental compensatory changes because transient knockdown of *Pink1* also shows similar results.

The mechanism(s) by which *Pink1* regulates survival has not been identified as yet. The localization of *Pink1* alone is complex. It has been reported to localize to the inner mitochondrial membrane basally and at the outer mitochondrial membrane following mitochondrial membrane depolarization (4, 6). It is suggested that *Pink1* can exist also in the cytoplasm (23, 40, 41). This last point is particularly intriguing given the fact that although the kinase activity of *Pink1* is critical for its protective effects when expressed, its mitochondrial localization sequence is not required either *in vitro* or *in vivo* in response to mitochondrial stress (23). This finding suggests that although *Pink1* may play a role in mitochondrial quality control, this may not be the primary pathway by which survival is mediated. Alternatively, *Pink1* may act on substrates in the cytoplasm to mediate survival as it is known that when *Pink1* is localized to the outer mitochondrial membrane, its kinase domain faces the cytoplasm (42). Interestingly, it was shown that stable knockdown of *Pink1* in cell culture system promotes autophagy. This autophagic event due to the absence of *Pink1* can be reversed by overexpression of WT *Pink1* or Δ*Pink1* (43). However, the link between autophagy and survival is unclear. In this regard, autophagy has been shown either to promote or inhibit death processes. Finally, *Pink1* has also been shown to phosphorylate *Trap1*, and this phosphorylation event is necessary for survival against oxidative stress (11). Whether this is critical in the adult DA system is unknown.

Interaction of Pink1 with Other Autosomal Recessive PD Genes, DJ-1 and Parkin—There are several common features of the three recessive PD genes *DJ-1*, *Parkin*, and *Pink1*. Loss of all of three genes has in some way been associated with mitochondrial deficits in the mouse tissue (3, 37, 44). Deficiencies also appear to mediate defects in DA uptake and turnover (19, 45, 46). These three genes are also associated with management of free radical damage (7, 9, 23, 34, 35). For example, several reports indicated that *DJ-1* expression protects and its loss sensitizes to oxidative inducers (8). Similar observations have been shown for *Parkin* and *Pink1* (23, 34, 35). In fact, *DJ-1* itself has atypical peroxyredoxin activity, was modified by reactive oxygen species (7), and was shown to regulate the stability of one of the master antioxidant regulator *Nrf2* (47). *Parkin* is also reported to induce genes such as *TFAM*, which is critical for mitochondrial biogenesis (48). How these parameters relate to the direct mechanism of cell-mediated survival is not known. Likewise, whether or not these three recessive genes act together to mediate survival *in vivo* remains to be established. As mentioned previously, ablation of all three recessive genes does not lead to DA neuron loss *in vivo* (39). This argues against the notion that these genes act in parallel to mediate basal DA survival *in vivo*. However, our present work supports the notion that modulation of either *DJ-1* or *Parkin* levels can compensate for the loss of *Pink1*. This is consistent with reports indicating that *Parkin* and *DJ-1* can also compensate for *Pink1*-mediated defects in the fly (49). Moreover, it supports a recent report that *DJ-1* acts in parallel with the *Pink1*/*Parkin* pathways (50).

Our observation that *Parkin* can compensate for *Pink1* deficiency is also interesting in light of the recent observations that *Pink1* is required for *Parkin* translocation to the mitochondria upon mitochondrial depolarization (17). Therefore, one interpretation of this finding is that *Parkin* acts downstream of *Pink1*, and thus the ability of *Parkin* expression to rescue *Pink1* deficiency make sense. However, there are some caveats regarding this interpretation. First, it is thought that *Pink1* is an absolute requirement for *Parkin* translocation. In this regard, our results demonstrating *Parkin*-mediated protection in the absence of *Pink1* are pertinent. The manner by which *Parkin* is mediating survival must be independent of its presence in the mitochondria. In addition, a mouse model defective in respiratory chain components which results in fragmented mitochondria and dissipation of membrane potential did not demonstrate *Parkin* accumulation at the mitochondria (38). They also found that mitophagy in these mice was not impacted in the presence or absence of *Parkin*, arguing against the role of *Parkin* in clearing defective mitochondria. Taken together, these observations call into some question whether or not mitochondrial quality control pathways mediated by *Pink1*/*Parkin* may be critical for DA survival. Much more work is clearly needed to address these essential questions. Nevertheless, our work clearly demonstrates that endogenous *Pink1* is an essential component that prevents neuronal loss in the adult SNc in response to environmental stress. We also provide evidence that modulation of either *Parkin* or *DJ-1* can protect even in the absence of *Pink1*, suggesting that *Pink1* is not a required factor for *DJ-1*/*Parkin*-mediated protection.

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