Role of the prolyl isomerase Pin1 in the pathogenesis of Parkinson's disease and neuroprotection by novel targeted compounds in pre-clinical animal models of the disease

Anamitra Ghosh

Iowa State University
Role of the prolyl isomerase Pin1 in the pathogenesis of Parkinson’s disease and neuroprotection by novel targeted compounds in pre-clinical animal models of the disease

by

Anamitra Ghosh

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Neuroscience

Program of Study Committee:
Anumantha G. Kanthasamy, Major Professor
Ann Smiley-Oyen
Arthi Kanthasamy
Marian L. Kohut
N. Matthew Ellinwood

Iowa State University
Ames, Iowa
2012

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ABSTRACT

Parkinson’s disease (PD) is a chronic and progressive neurodegenerative disorder named by the French neurologist Jean-Martin Charcot, after the British physician James Parkinson who first described the disease as “Shaking Palsy”. Pathologically, PD is characterized by a progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), a marked reduction of dopamine in the striatum, the presence of ubiquitin and α-synuclein positive cytoplasmic inclusions known as Lewy bodies, and depigmentation of the locus cereleus. The prevailing theory regarding processes that are likely to account for progressive degeneration of the dopaminergic neurons in the nigrostriatal axis involves on mitochondrial dysfunction, oxidative stress, excitotoxicity and neuroinflammation. Of those, neuroinflammation and oxidative stress have gained the most focus recently. Research over the last decade has provided extensive evidence that the sustained microglial and astroglial neuroinflammatory responses cause progressive and delayed dopaminergic neurodegeneration. However, the mechanisms by which glial cells activation and subsequent inflammation lead to dopaminergic neurodegeneration remain poorly understood. Development of effective therapeutic approaches to halt the disease progression of PD is of paramount importance. My objective in this Ph.D. thesis work was to characterize important signaling molecules activated in neuroinflammation mediated neurodegenerative pathways as well as characterize of novel compounds in a pre-clinical mouse model of PD. Protein interacting with never in mitosis – A (Pin1) is a peptidyl-prolyl isomerase, that specifically recognizes phosphorylated serine or threonine residues immediately preceding proline (pSer/Thr-Pro) in a subset of proteins in which it isomerizes the cis/trans conformation of the
peptide bond. High expression levels of Pin1 in terminally differentiated and post mitotic neurons suggest that it plays an important role in neurons, except in cell cycle regulation and proliferation. Recently, Pin1 overexpression was shown to facilitate formation of α-synuclein inclusions in a cellular model of α-synuclein aggregation. Pin1 was also localized in Lewy bodies in PD patients. But the level, activity, and role of Pin1 in the pathogenesis of PD are incompletely known. We hypothesized that Pin1 is differentially activated in neuronal and glial cells of the nigral dopaminergic system, and that it regulates NF-κB-mediated sustained neuroinflammatory processes in cell culture and in an animal model of Parkinson’s disease. Herein, we demonstrate for the first time that there is a dopaminergic neuron specific upregulation of Pin1 in human postmortem PD brain sections as well as in cell culture and animal models. We observed a rapid increase in Pin1 expression in both the 1-methyl-4phenyl pyridinium (MPP⁺)-treated cell culture and in 1-methyl-4-phenyl-1-2-3-6-tetrahydrotridine MPTP treated mice. Also, Pin1 acts as an important pro-apoptotic factor in the selective degeneration of dopaminergic neurons. Importantly, pharmacological inhibition of Pin1 attenuates MPTP-induced Pin1 expression in vitro and in vivo and protects against MPTP-induced neurodegeneration in the nigrostriatal axis. We also demonstrate for the first time that, microglia and astrocytes express Pin1 and that there is a strong association between Pin1 and NF-κB p65 in BV2 microglial cells. Recent studies demonstrated that the promoter regions of proinflammatory molecules contain the DNA binding site for NF-κB. We have shown that Pin1 and NF-κB p65 inhibition by Juglone leads to attenuation of glial cells activation and subsequent reduction of proinflammatory reactions in cell culture and in an animal model of PD. While characterizing the role of Pin1 in the pathogenesis of PD, we also tested the efficacy of novel compounds for protection of dopaminergic neurons in a mouse
model of PD. We demonstrate that the novel compound diapocynin, a metabolite of apocynin, blocks MPTP-induced activation of microglial and astroglial cells, thus inhibiting the inflammatory and oxidative stress processes in MPTP-treated mice. Diapocynin also protects the nigrostriatum against MPTP toxicity. The final chapter of this work characterizes the anti-inflammatory and neuroprotective properties of mito-apocynin, a mitochondria targeted compound in the MPTP mouse model of PD. Mitoapocynin prevents the behavioral impairments and dopamine loss caused by MPTP-induced toxicity. We have shown that mito-apocynin protects the nigrostriatum by attenuating glial cell mediated neuroinflammation and oxidative stress. Collectively, the research described herein characterizes the novel and important roles of Pin1 in the neuroinflammation and pathophysiology of Parkinson’s disease, as well as establishes the efficacy of novel compounds in protection of the nigrostriatum in a pre-clinical mouse model of Parkinson’s disease.
CHAPTER I

GENERAL INTRODUCTION

Thesis Layout and Organization

The alternative format was chosen for writing this dissertation as it consists of manuscripts that are either under peer review or being prepared for submission. The dissertation contains a general introduction, four research papers, a general conclusion that discusses the overall findings from all the chapters and acknowledgement. The references for each manuscript chapter are listed at the end of that specific section. References pertaining to the background and literature review as well as those used in general conclusion section are listed at the end of the dissertation. The General Introduction (Chapter I) briefly provides the current knowledge on Parkinson’s disease and describes an overview of the research objective. The Background and Literature Review I provide background information on Parkinson’s disease (PD), pathological features of PD, physiology and chemistry of dopamine, genetics of PD and apoptosis in PD, MPTP mouse models of PD and role transcription factor NF-κB in neurodegeneration. It also has the current knowledge of peptidyl prolyl cis/trans isomerase, Pin1 in cancers and neurodegenerative disorders, and provides an overview of the research objectives pertaining to chapters II and III. Background and Literature Review II summarize the current evidences implicating a pathogenic role for reactive gliosis in dopaminergic neurodegeneration, role of oxidative stress and inflammation in PD pathogenesis, and current therapeutic approaches against progressive
neurodegeneration in PD. Also, it provides an overview of research objectives pertaining to chapter IV and V.

This dissertation contains the experimental data and results obtained by the author during his Ph.D. study under the supervision of his major professor Dr. Anumantha G. Kanthasamy at Iowa State University, Department of Biomedical Sciences.

**Introduction**

Parkinson’s disease (PD) is the most common movement associated neurodegenerative disorder affecting balance, movement, flexibility and coordination. Pathologically it is characterized by the loss of dopaminergic neurons in the substantia nigra region of the midbrain and its terminal in the striatum, the presence of cytoplasmic inclusions (Lewy bodies) in degenerating dopaminergic neurons in the nigra and activation of glial cells (Dauer and Przedborski, 2003). The pathophysiological mechanisms behind nigral neurodegeneration in PD are still not clear. Expanding research had shed significant light in understanding PD etiology and molecular pathogenesis. In the last two decades, researchers focused mainly on mitochondrial dysfunction, oxidative stress, apoptosis and neuroinflammation to elucidate the disease mechanisms. Also, numerous studies conducted in *in vitro* and *in vivo* models of PD suggest the roles played by these mechanisms result in irreversible and selective degeneration of dopaminergic neurons (Hunot and Hirsch, 2003; Jenner, 2003). Epidemiological studies implicate both environmental factors and genetic predisposition as risk factors contributing to PD (Di Monte, 2003).

Recent reports suggest an important role of neuroinflammation of nigrostriatal degeneration in PD patients (Dauer and Przedborski, 2003; Gao et al., 2003). Activation of glial cells (microglia and astrocytes) is evident in close proximity to the damaged or dying neurons in
substantia nigra. Elevated levels of inflammatory molecules such as nitrite and inducible nitric oxide synthase are found in substantia nigra of PD patients (Qureshi et al., 1995; Hunot et al., 1996). Additionally, a variety of proinflammatory cytokines such as tumor necrosis factor (TNF-α), interleukin -1β (IL-1β), interleukin 6 (IL-6), eicosanoids and other immune neurotoxins are found either in CSF or affected brain regions in PD (Mogi et al., 1994; Bessler et al., 1999; Nagatsu et al., 2000). A recent report demonstrated nuclear factor-κB (NF-κB), a transcription factor that is required for the transcription of proinflammatory molecules, is activated in substantia nigra of PD patients and MPTP-treated mice (Ghosh et al., 2007). Despite all these findings, the mechanism of glial cell activation and production of cytokines in response to neuronal toxicity and what activates the neurons and glial cells to promote dopaminergic neuronal degeneration is yet unknown. Reversible phosphorylation on Ser/Thr – Pro (S/T-P) motifs regulated by Pro-directed protein kinases and phosphatases is an important molecular switch in controlling various cellular processes (Gothel and Marahiel, 1999). Since the discovery of protein interacting with never in mitosis – A (Pin1), much is known about the conformational importance of Pro-directed kinases in cellular signaling (Uchida et al., 1999). Pin1 is unique among the peptidyl-prolyl isomerases in that they specifically recognize phosphorylated serine or threonine residues immediately preceding proline (pSer/Thr-Pro) in a subset of proteins and isomerizes the cis/trans conformation of peptide bond (Joseph et al., 2003; Lu, 2004). Recently Ryo et al., 2006 reported that Pin1 overexpression facilitates formation of α-synuclein inclusions in a cellular model of α-synuclein aggregation and Pin1 also localizes in Lewy bodies in PD patients. Previous reports indicate the role of Pin1 in controlling several transcription factors that mediate induction of iNOS (Gothel and Marahiel, 1999; Shen et al., 2005). But its level, activity and
role in the inflammatory responses in the pathogenesis of PD are completely unknown. The present study characterizes the novel molecule Pin1 in cell culture animal models of PD as well as investigates the pathological roles of Pin1 in oxidative stress and inflammation mediated dopaminergic neurodegeneration of PD. In addition, this study also investigates the efficacy of novel compounds in protecting nigrostriatal axis in MPTP mouse model of PD.
Background and Literature Review I

This review section will provide background information on Parkinson’s disease (PD), pathological features of PD, physiology and chemistry of dopamine, genetics of PD, apoptosis in PD, MPTP mouse models of PD, and role of transcription factor NF-κB in neurodegeneration. Also discussed is the current knowledge of peptidyl prolyl cis/trans isomerase, Pin1 in cancers and neurodegenerative disorders.

Parkinson’s disease

Parkinson’s disease (PD) is a progressive neurodegenerative disorder that affects more than 1 million individuals over the age of 60 within the United States (Jankovic and Stacy, 2007). According to recent article, the number of new cases increased by about 50,000 annually (Dauer and Przedborski, 2003). Although PD is an age-related disorder affecting nearly 3% of people over 60 years and 4-5% people over 85 years of age, nearly 10% of PD cases are under 40 years of age (Mizuno et al., 2001). Epidemiological studies suggest that sporadic PD cases (90%) are predominant with late onset whereas, remaining 10% cases are characterized by early onset and these cases mainly occur in familial clusters (Mizuno et al., 2001; Tanner, 2003). The familial or early onset PD had been linked with mutations in several genes such as parkin, ubiquitin C-terminal hydrolase L1, α-synuclein, leucine rich repeat kinase 2(LRRK2), PINK-1 or DJ1 (Gwinn-Hardy, 2002; Tang et al., 2006; Weng et al., 2007; Bonifati et al., 2008). The cause of sporadic PD or non-familial PD are yet unknown, but several reports suggest environmental toxins, genetic factors, mitochondrial dysfunction, apoptosis, oxidative stress and neuroinflammation could be the possible factors behind the PD neurodegeneration (Schapira, 1994; Ben-Shachar et al., 1995; Hoehn and Yahr, 1998; McGeer et al., 2001). Among the environmental toxins, infectious agents,
pesticides, herbicides and heavy metals have been implicated in the pathogenesis and progression of the disease (Mizuno et al., 2001). Recent investigations now stressed on inflammation and oxidative stress to be the central players in the pathogenesis of PD.

**Clinical features of Parkinson’s disease**

PD has been considered as a motor disease and the motor symptoms include tremor at rest, bradykinesia, rigidity, loss of postural balance. Among these symptoms tremor, bradykinesia and rigidity are the early symptoms, whereas abnormalities in postural balance appear late. Besides these cardinal features certain other motor symptoms have also been reported in PD like difficulties in getting up from the chair and turning in bed, a shuffling gait, micrographia (small or cramped handwriting), decreased arm swing, difficulty in cutting food and feeding, reduced hygiene, and a general drag in the activities of daily living (Jankovic, 2008). Cognitive impairment, depression and dementia have also been reported in PD patients and appearance of these symptoms is dependent on factors like age, the presence of Lewy bodies, co-occurrence of AD, level of progression of the disease, and gender (Bunting-Perry, 2006; Welge-Lussen et al., 2009). Additionally, PD patients have dramatically greater chances of developing dementia when compared to age matched controls (Aarsland et al., 2001; Farlow and Cummings, 2008).

Along with cardinal features and cognitive deficits, there is an increasing awareness of the non-motor aspects of PD such as insomnia (Double et al., 2003), sleeping disorders, anxiety, psychosis and mania, alcohol or other substances abuse, and sexual disorders (Schneider et al., 2008; Park and Stacy, 2009, Simuni, 2009 #38). Additionally, olfactory deficits have also been observed in PD patients very frequently irrespective of disease severity and duration (Double et al., 2003).
Pathological features of Parkinson’s disease

Primary pathological feature of PD is the loss of dopaminergic neurons in the substantia nigra (SN) region of the midbrain and its terminal in the striatum (Dauer and Przedborski, 2003). This loss of dopaminergic neurons causes most of the motor symptoms of PD. Surprisingly, the dopaminergic neurons of the adjacent ventral tegmental area (VTA) are relatively spared of degeneration. But, neuropathology of PD revealed that the neurodegeneration not only involves the dopaminergic neurons in SN, but also reaches to the dorsal motor nuclei of the hypoglossus and vagus nerves, dorsal part of raphe nucleus as well as the anterior olfactory nucleus and olfactory bulb, before it extends to the mesencephalic areas which include SN (Braak et al., 2003). Beside dopaminergic neurodegeneration, degeneration of serotonergic neurons in raphe nuclei and norepinephrinergic neurons in the locus coeruleus also occur (Braak et al., 2003). In the following section, we will briefly discuss an overview of the major dopaminergic systems in the brain, the basal ganglia and its functional anatomy, and some of the clinical aspects of PD pathology.

Major dopaminergic systems of the brain

In the brain, different dopaminergic pathways ascend from the following major nuclei – the mesencephalon (midbrain); the substantia nigra pars compacta (SNpc), the ventral tegmentum area (VTA) and the hypothalamus (Van den Heuvel and Pasterkamp, 2008). These nuclei are the major source of four major dopaminergic pathways such as the nigrostriatal pathway, mesolimbic pathway, mesocortical pathway and the tuberoinfundibular pathway. The nigrostriatal pathway is the largest among them, comprising of 70% of total central nervous system (CNS) dopaminergic neurons which synthesizes nearly 80% of brain dopamine (Van den Heuvel and Pasterkamp, 2008). This pathway starts from the SNpc of
ventral midbrain and projects to the dorsal striatum, ventral striatum and cerebral cortex. The dopaminergic neurons in the SNpc are very densely packed (hence the term *pars compacta*) and they contain neuromelanin, a dark pigment (hence the term *nigra*). These pigmented dopaminergic neurons mainly degenerate in PD. The serotonergic inputs from the medial and dorsal raphae nuclei and noradrenergic input from the locus coeruleus comes to the SNpc which in turn sends dopaminergic efferents to other brain regions (Rommelfanger et al., 2007). As the nigrostriatal pathway is connected to the dorsal sensorimotor striatum, it controls with motor function. The mesolimbic pathway ascends from the VTA region and projects not only to ventral striatum and nucleus accumbens, but also to other brain regions such as hippocampus and amygdale. The mesolimbic pathway plays an important role in emotion, motivation and addiction. The mesocortical pathways ascend from the VTA region and projects to cerebral cortex and it plays an important role in complex cognition (Bjorklund and Dunnett, 2007; Van den Heuvel and Pasterkamp, 2008). The tuberoinfundibular pathway arises in the hypothalamus and ends in the pituitary, where it inhibits prolactin secretion (Bjorklund and Dunnett, 2007).

**Dopamine neurochemistry**

Dopamine (DA) - the neurotransmitter produced by the dopaminergic neurons in the SNpc is similar to other catecholamines – noradrenaline (NAd) and adrenaline (Ad). The precursor amino acid of all these neurotransmitter is tyrosine. Tyrosine is converted to 3, 4-dihydroxy-L-phenylalanine (L-DOPA) by tyrosine hydroxylase (TH), the rate limiting enzyme in the biosynthesis of dopamine. In the next step, L-DOPA is decarboxylated to DA by aromatic L-amino acid decarboxylase/dopamine decarboxylase (DDC). Dopamine can be synthesized both in and outside the brain due to DDC’s presence in both the central nervous
system (CNS) and its periphery. But, DA cannot cross the blood brain barrier (BBB) and therefore has to be synthesized from L-DOPA inside the brain. In the noradrenergic neurons, DA is further converted to noradrenaline by dopamine β-hydroxylase (DBH). DA after synthesized get released in to the synaptic junction through dopaminergic neuron terminals but it can be inactivated by reuptake into the pre-synaptic neurons by specialized membrane monoamine transporters, including the DA transporter (DAT) and noradrenaline transporter – the vesicular monoamine transporters (VMAT) and concentrated into synaptic vesicles. Intracellularly, dopamine is degraded by monoamine oxidase enzyme to dihydroxyphenylacetic acid (DOPAC) which is further degraded to homovanillic acid (HVA) by catechol-o-methyl transferase (COMT). Both these catabolic products are also quantified in studies pertaining to neuroprotection in PD because the biological levels of these metabolites is also an indicator of the neuroprotection by the neuroprotective compounds in question (Kandel and Squire, 2000).

**Figure 1. Dopamine biosynthesis pathway.** Source: (Hare and Loer, 2004)

**Neuroanatomy of basal ganglia**

The basal ganglia is a critical part of extrapyramidal motor system consisting of striatum, external (GPe) and internal (GPi) pallidal segments, subthalamic nucleus and substantia nigra (comprising of pars compacta – SNpc and pars reticularis – SNpr). The striatum is made up of two regions – caudate nucleus/putamen (neostriatum) and ventral
striatum. Substantia nigra of ventral midbrain has cell bodies in the SNpc and axons in the Striatum region. The neostriatum and nucleus accumbens send their output directly into the output nuclei – the *globus pallidus internal segment* (GPi) and SNpr or indirectly with intermediate stops in *globus pallidus external segment* (GPe) and sub-thalamic nucleus (STN). In turn, the GPi and SNpr send their output back to cerebral cortex via the thalamus. The pathways consist of two subtypes. The first one ‘direct pathway’, consists of monosynaptic connections with GPi and the SNpr. The other one is called the indirect pathway (GABAergic neurons – Enkephalin) and it is made of polysynaptic connections traversing the GPe segment and connecting with the GPi/SNpr via the subthalamic nucleus (STN). The direct pathway is mediated by excitatory D1 receptors and in this pathway medium spiny neurons project from striatum and directly inhibit the output nuclei GPi and SNpr. The medium spiny neurons inhibit these output nuclei because the output of the GPi and SNpr to the thalamus is inhibitory, which ultimately leads to a less inhibitory effect on the cerebral cortex, via the thalamus. The indirect pathway is mediated by inhibitory D2 receptors and in this pathway medium spiny neurons send an inhibitory GABAergic projection towards the GPe. Because of this inhibitory effect, the medium spiny neurons were inhibited and the GPe becomes free and can normally project its output to its target nuclei. The STN receives inhibitory GABAergic input from GPe as well as inhibitory signal directly to the SNpr. The STN also sends excitatory input to the SNpr and GPi, which becomes inhibited by the GPe. Consequently, the cerebral cortex also sends a direct excitatory influence on the STN. Therefore, indirect pathway excites the output nuclei, which in turn leads to an inhibition of the thalamic/cortical output. Collectively, the indirect and the direct pathways have a contradictory effect. The direct pathway facilitates motor or cognitive
programs from cortical areas, whereas the indirect pathway inhibits these programs (Albin et al., 1989).

**Figure 2. Direct and Indirect pathways.** A. The supplementary motor area (SMA) is activated through the 'direct' pathway as follows. (1) Corticostriate fibers from the sensorimotor cortex activate GABAergic neurons in striatum having D$_1$ receptors tonically facilitated by nigrostriatal inputs. (2) The activated striatal neurons inhibit medial pallidal (GPM) neurons (3) with consequent disinhibition of ventral lateral nucleus (VLN) thalamocortical neurons (4) and activation of SMA (5), which both modifies ongoing corticostriate activity and initiates impulse trains along corticospinal (CST) and corticoreticular (CRST) fibers.

B. (1) Corticostriate neurons from the sensorimotor cortex strongly activate GABAergic neurons (2) in the striatum that synapse on others (3) in the lateral pallidal segment (GPL). The double effect is disinhibition of the subthalamic nucleus (STN). STN discharges
strongly (4) on to the GABAergic neurons of the medial pallidal segment (GPM); these in turn discharge strongly (5) into the ventral lateral nucleus (VLN) of thalamus, resulting in reduced output along thalamocortical fibers (6) traveling to the supplementary motor area (SMA). Inputs (7) from SMA to corticospinal and corticoreticular fibers (CST, CRST) become progressively weaker, with pathetic consequences for initiation and execution of movements. (Modified from “Clinical Neuroanatomy and Neuroscience- Fifth Edition” by MJ Turlough FitzGerald, Gregory Gruener, Estomih Mtui)

**MPTP mouse model of Parkinson’s disease**

The use of animal models to gain insight into human diseases and the potential treatment is extremely valuable. As opposed to isolated cell culture (*in vitro*) models, the use of a living animal as a model system is more reliable and productive for studying multifactorial diseases like PD. PD is a uniquely human disorder and the etiology of PD is not known completely. There are no animal models which can mimic the exact features of PD in human. However, some of the pathological symptoms can be reproduced using different toxins such as methamphetamine, 6-hydroxydopamine, rotenone, paraquat and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in animal models (Langston and Ballard, 1983; Chiba et al., 1984; Heikkila et al., 1984; Finnegan et al., 1987; Betarbet et al., 2000; Deumens et al., 2002; McCormack et al., 2002). Since the discovery of MPTP in the early 1980s, its ability to degenerate nigrostriatum dopaminergic neurons after systemic administration in a number of different species, led to its recognition as a potent Parkinsonian neurotoxins (Langston and Ballard, 1983; Schneider et al., 1987).

*Conversion of MPTP to MPP+, the neurotoxic substance*

In non-human primates, MPTP administration develops clinical features very similar to human PD (Schneider et al., 1988; Schneider, 1989). MPTP is a protoxin, highly lipophilic
molecule which can easily cross the blood brain barrier within minutes after its systemic
administration (Markey et al., 1984). Once in the brain, it is converted to 1-methyl-4-phenyl-
2,3-dihydropyridinium (MPDP⁺) via monoamine oxidase b (MAO-B) in astroglial cells and
serotonergic cells (Chiba et al., 1984; Kitahama et al., 1991). However, MPDP⁺ is an
unstable molecule which immediately converted to 1-methyl-4-phenylpyridinium (MPP⁺),
the actual neurotoxin (Chiba et al., 1985; Peterson et al., 1985). MPP⁺ then released from
glial cells and enters to dopaminergic neurons via dopaminergic transporter (DAT). MPP⁺ is
far less lipophilic than MPTP and is unable to cross the blood-brain barrier (Bezard et al.,
1999). Once inside the neuron, MPP⁺ is passively transported to mitochondrial matrix
(Ramsay and Singer, 1986). Intramitochondrial MPP⁺ inhibits oxidative phosphorylation by
imparing ADP stimulated oxygen consumption and the multi-subunit enzyme Complex I
(NADH-ubiquinone oxidoreductase).

![Chemical Structures of MPTP, MPDP⁺, and MPP⁺]

**Figure 3. Conversion of MPTP to MPP⁺ (Tipton et al., 2001)**

Report have also shown that MPP⁺ binds to Complex I and blocks the terminal step of
electron transport transfer from the highest potential iron-sulfur cluster of Complex I,
resulting in impaired synthesis of ATP (Ramsay et al., 1987). Additionally, Complex I
inhibition by MPP⁺ also results in an increased production of reactive oxygen species (ROS).
Using MPTP mice model of PD, researchers also demonstrated oxygen-dependent formation of ROS. Both impaired synthesis of ATP and production of ROS lead to stimulation of apoptotic pathways which ultimately lead to dopaminergic neurodegeneration.

Different MPTP dosing schedule

Among various and commonly used neurotoxins, MPTP is the most popular one as, use of MPTP is very simple, systematic admistration produces PD like symptoms, it can mimic the clinical PD features in rodents and primates more closely than any other neurotoxins. Depending on doses and time of administration, MPTP mouse model of PD is of three types - acute, sub-acute and chronic. In acute regimen, 4 doses of MPTP (ranges from 15mg/kg to 20mg/kg) at 2 h interval are administered via intraperitoneally (i.p.), sub-acute MPTP paradigm consists of 3-5 doses of MPTP (ranges from 20mg/kg/dose to 30mg/kg/dose) via i.p. with single dose per day and in chronic model of MPTP, mice receive 3 doses of MPTP (ranges from 20mg/kg to 30 mg/kg) per week for 5 weeks via i.p. along with probenecid (sub-cutaneous) treatment. While all these paradigms rely on same initial mechanism of neurotoxicity, the modes of cell death are different in each model. Following acute administration of MPTP, death of DA neurons occurs primarily via non-apoptotic or necrotic pathways (Jackson-Lewis et al., 1995; Przedborski and Vila, 2003), whereas, in sub-acute and chronic treatment, neurons appear to die via apoptosis (Tatton and Kish, 1997). In both theses models, the loss of dopamine takes place almost immediately after treatment, while the neuronal degeneration occurs in a slower rate. One big shortcoming of MPTP mouse model is lack of progressiveness in dopaminergic neurodegeneration. Following MPTP treatment, death of DA neurons starts within 12h after the last injection and is completed by 4-5 days post-treatment (Jackson-Lewis et al., 1995), where as in PD, slow
progressive dopaminergic neurodegeneration occurs. Besides loss of dopaminergic neurons in nigrostriatum, the neuropathology of PD is also characterized by the presence of cytoplasmic inclusion known as lewy bodies in surviving neurons (Shults, 2006). However, chronic MPTP mouse model and mice administered with MPTP via osmotic pumps do show lewy bodies (Fornai et al., 2005; Jin et al., 2005). But, in acute or sub-acute MPTP intoxicated mice, lewy bodies could never be seen. Different factors such as age, weight, strain and gender backgrounds have been reported to influence reproducibility and the extent of MPTP-induced damages in mice (Giovanni et al., 1991; Hamre et al., 1999; Staal and Sonsalla, 2000). Moreover, different strains of mice and even within a given strain obtained from different vendors can exhibit different sensitivity to MPTP (Heikkila et al., 1989). However, these different sensitivities have been reported to be transmissible as an autosomal dominant trait (Hamre et al., 1999). The most severe neurotoxicity of MPTP was observed in the C57BL/6 mice strain in comparison to the other mice strains as the clearing of MPTP and MPP⁺ was less rapid in the brains of the C57BL mice when compared to other mice strains (Schwarting et al., 1999; Sedelis et al., 2000). Consistency in the reproducibility of MPTP-induced neurotoxicity is best observed in the mice in 8-12 weeks of age weighing about 28g body weight, indicating that it is important to investigate on the mice falling under these physiological condition for MPTP studies. MPTP mice model also reproduce the behavioral
deficits associated with PD. Muscle rigidity, tremor, gait and postural imbalances are the most common symptoms in PD patients (Meredith, 1958; Hattori and Kubo, 2007). However, based on the MPTP dosing regimens, behavioral data are often widely differed in mice (Chiueh et al., 1984).

**Genetics of PD**

Although the rare early-onset PD accounts for approximately 5 to 10% of the total PD cases, significant advances in understanding the mechanisms of disease pathogenesis have been made in the past two decades with the identification of distinct gene loci at which pathogenic mutations are associated with parkinsonism. Several genetic mutations have been described in the rare familial early onset PD. The most prevalent genes conclusively associated with PD are α-synuclein, leucine-rich repeat kinase (LRRK2), PTEN induced putative kinase (PINK1), Parkin and DJ-1(PARK7) (Litvan et al., 2007; Cookson and van der Brug, 2008). Briefly, in this section, the mutation, pathology and symptoms associated with a-synuclein have been discussed.

Alpha-synuclein is a small presynaptic protein in the CNS (140kDa) encoded by the α-synuclein gene SNCA. It is characterized by the presence of repetitive and imperfect repeats of KTKEGV amino acids distributed throughout the amino terminal and C terminal halves (Maroteaux et al., 1988). Exact physiological function of α-synuclein is not known, though, it is suggested that α-synuclein significantly interacts with tubulin and may have a potential microtubule – associated protein like function. α-synuclein may also function as a molecular chaperone protein, because, its N-terminal sequence (1-61) possess about 40% homology with the 14-3-3 proteins, which are well understood chaperone proteins. Additionally, it has been demonstrated α-synuclein interacts with cholesterol and
sphingomyelin containing vesicles and that interaction leads to its stabilization (Kamp and Beyer, 2006). Two mutations in α-synuclein – A30P and A53T were identified in familial PD in a German family and a Greek-Italian family, respectively. For last few years, mice over-expressing A30P and A53T mutants serve as important genetic models of PD. In the PD brain, α-synuclein is detected in lewy bodies in neurons of the brain stem and cortex and aggregates of α-synuclein are a major protein component of these structures (Lee, 2003). Previous reports demonstrated the mutation of α-synuclein that leads to develop b-sheets rich structure and polymerize into oligomers (Lee, 2003) and this oligomeric α-synuclein may be toxic to the dopaminergic neurons (Conway et al., 2000). This toxicity could also be attributed to the auto-oxidation of dopamine on protofibrillar α-synuclein aggregation (Conway et al., 2001). Also, these oligomers are highly resistant to prolonged boiling, high SDS concentrations and treatment with urea (Perrin et al., 2001). These oligomeric fibrils which accumulate within the cell bodies and neurites as Lewy bodies and Lewy neuritis, also cause damage to ER, mitochondria and other organelles. Overexpression of α-synuclein in dopaminergic cells treated with dieldrin (a pesticide) resulted in susceptibility of the dopaminergic neurons to apoptosis, suggesting possible role of α-synuclein in the idiopathic PD (Sun et al., 2005). In contrast, over-expression of human wild-type (WT) α-synuclein resulted in attenuation of dopaminergic neuronal apoptosis (Kaul et al., 2005a). Recently, we have shown that human WT α-synuclein interacts with pro-apoptotic proteins - PKCδ and attenuates dopaminergic apoptosis and α-synuclein negatively regulates PKCδ in dopaminergic neurons by reducing p300 Histone acetyl transferase (HAT) activity (Jin et al., 2011). Aging is another factor that may contribute to the α-synuclein toxicity in the age
related accumulation of oxidative and nitrative α-synuclein aggregation (Lee and Trojanowski, 2006). Additionally, amyloid b peptide (Ab) which is responsible for aggregation in Alzheimer’s disease, promotes α-synuclein aggregation. It has been recently demonstrated that α-synuclein is directly transmitted from neuron overexpressing the protein to adjacent healthy embryonic stem cells in culture or in transgenic animals (Desplats et al., 2009). Additionally, misfolded α-synuclein aggregates mimic to the pathogenic prion protein conformer (PrP^{sc}) which could lead to aggregation of native protein (Ferreon et al., 2009). Lentivirus mediated overexpression of α-synuclein led to neurotoxicity with preferential loss of dopaminergic neurons (Cooper et al., 2006). From the existing findings, it is not clear if α-synuclein plays a pro-survival or an anti-survival role in dopaminergic neurons. Extensive investigations are required to give a better picture of α-synuclein’s roles in PD pathology.

**Apoptosis in PD**

Rapidly growing evidence indicates that mitochondrial dysfunction leads to consequent loss of energy production (ATP), oxidative stress and associated apoptosis. Recent observations have found changes in the mitochondrial respiratory chain complex I in the necropsy samples of PD patients and in rotenone, paraquat or MPTP induced PD models that trigger the dopaminergic degeneration (Greenamyre et al., 1999; Greenamyre et al., 2001; Ayala et al., 2007).

Among the protein complexes that make up the electron transport chain, Mitochondrial complex I is one of them. It resides in the inner membrane of the mitochondria. Inhibition of complex I results in an interruption in the flow of electrons along the electron transport chain, which leads to inappropriate synthesis of ATP. Consequently, resultant energy deficiency as well as oxidative stress and apoptosis pathways become gets
activated and causes neurodegeneration (Knott et al., 2008). Inhibition of Complex I leads to the leaking of electrons from the electron transport chain and results in partial reduction of oxygen to superoxide which further damages and impairs the mitochondrial function and makes the cell vulnerable to the activation of apoptotic pathways (Casetta et al., 2005; Zhou et al., 2008). Apoptosis is a predominant mechanism which is morphologically characterized by cell shrinkage, cytoplasmic blebbing and involution. Apoptosis event is also involved in breaking up of cells into smaller apoptotic bodies that are removed by phagocytosis without the involvement of inflammatory responses. Although, it is not completely known whether the pattern of dopaminergic neurodegeneration follows apoptosis or necrosis, apoptosis is believed to be a dominant mechanism of cell death during PD (Anglade et al., 1997; Tatton et al., 2003). Growing evidence from necropsy investigations of PD patients also suggests apoptosis could be a primary form of cell death during PD. But as apoptotic pathways are very long process, it occurs over several years and subsequently, the dying cells are cleared rapidly. Examination of post-mortem brain tissues of PD patients provides the most information on apoptosis as a predominant mechanism of dopaminergic neuronal cell loss. Several reports have demonstrated that the dying neurons express Bcl-2 family of pro- and anti-apoptotic proteins and caspases, and these results suggest that apoptotic mechanisms are the predominant pathways in the dying neurons (Mogi et al., 1996; Tortosa et al., 1997). Also, apoptotic mechanisms are initiated in the MPTP and other neurotoxins models of PD depending on the dosing regimen of those toxins. For example, sub-acute and chronic low-dose of MPTP triggers the apoptotic cell death pathways. A recent study has demonstrated that the Bax ablated mice are resistant in the MPTP mouse model of PD (Vila et al., 2001). Similarly, over-expression of anti-apoptotic Bcl-2 is also protective in a MPTP model (Cao et
al., 2006). The tumor suppressor protein p53 regulates Bax protein expression and that p53 activity and expression are upregulated in the MPTP-treated mice (Trimmer et al., 1996). Similarly, p53 null mice are also resistance to MPTP-induced dopaminergic neurodegeneration (Duan et al., 2002). Additionally, MAPKs and JNK ablated mice are also resistant to MPTP toxicity (Saporito et al., 1999; Saporito et al., 2000).

Permeabilization of the mitochondrial outer membrane (MOMP) by the pro-apoptotic members of the Bcl-2 family of proteins initiates the intrinsic pathway of apoptosis, resulting in the release of apoptogenic factors from the innermembrane space into the cytoplasm. Cytochrome c is one of these factors which binds to APAF-1 and causes a conformational change to APAF-1 which leads to its oligomerization and recruitment of caspase-9 to form a complex called apoptosome. Consequently, caspase-9 is activated and in turn activates and cleaves the executioner caspase-3 and caspase-7. Activated executioner caspases further activates the apoptotic cascade by cleaving key substrates to produce the biochemical and cellular events that are typically associated with apoptosis (Lin et al., 2010; Kiechle et al., 2002). Recently, we have discovered that PKC-\(\delta\), one of the key substrates of caspase-3 activated in MPTP and other toxin related models in PD (Zhang et al., 2007a). Previously we have shown that PKC\(\delta\) is proteolytically cleaved by active caspase-3 to generate a constitutively active catalytic fragment of PKC\(\delta\) which mediates further downstream apoptotic signaling in dopaminergic neurons (Kitazawa et al., 2003; Kaul et al., 2005b; Afeseh Ngwa et al., 2009).

**Pin1**

Protein phosphorylation is one of the most important regulatory mechanism in both prokaryotic and eukaryotic organisms (Stock et al., 1989; Barford and Neel, 1998).
Regulatory phosphorylation generally occurs on serine, threonine or tyrosine residues in eukaryotic proteins and histidine, arginine and lysine in prokaryotes. Reversible phosphorylation on Ser/Thr – Pro (S/T-P) motifs regulated by Pro-directed protein kinases and phosphatases is an important molecular switch in controlling various cellular processes (Gothel and Marahiel, 1999; Lu, 2000; Jager et al., 2006). Proline-directed kinases, such as cyclin-dependent protein kinases, extracellular signal-regulated kinases, stress-activated protein kinases/c-Jun-N-terminal kinases, p38 MAP kinases, glycogen synthase kinase – 3, Polo-like kinases and some of the Ser/Thr phosphatases, phosphorylate or dephosphorylate such motifs to regulate cellular signaling (Sudol et al., 2001; Shaw, 2007). Recent studies demonstrated that Ser/Thr-Pro phosphorylated proteins undergo post-phosphorylation conformational modifications by the phosphorylation-specific peptidyl-prolyl cis/trans isomerase (PPIase) (Lu et al., 2007; Lu and Zhou, 2007). Since the discovery of protein interacting with never in mitosis – A (Pin1), a lot has been learned with respect to the conformational importance of the Pro-directed kinases in cellular signaling (Uchida et al., 1999; Fujimori et al., 2001). Pin1 is a peptidyl-prolyl isomerases but it is unique among PPIase, in that, it specifically recognizes phosphorylated serine or threonine residues immediately preceding proline (pSer/Thr-Pro) in a subset of proteins, and isomerizes the \textit{cis/trans} conformation of the peptide bond (Joseph et al., 2003; Lu, 2004).

**Structure of Pin1**

Pin1 is an 18kDa protein and it has a two domain structure that comprised of N-terminal WW domain (named after two invariant Trp residues) and C-terminal PPIase domain. The WW domain binds to specific pSer/Thr-Pro motif and PPIase domain isomerizes specific pSer/Thr-Pro motifs to regulate protein functions by controlling their
conformation (Lu et al., 1999; Zhou et al., 2000). The two domains are joined together by a flexible linker. Molecular modeling study finds the high homology of Pin1 across human, rat and mouse. Various nuclear magnetic resonance (NMR) analyses revealed that Pin1 dynamically binds to its substrates and regulates their conformation (Jacobs et al., 2003; Pastorino et al., 2006; Namanja et al., 2007). Still, it is not clear why Pin1 binds only to specific pSer/Thr-Pro residues in certain proteins. However, different articles speculated that the sequence that is important for the Pin1 binding specificity is located on the WW domain at an intrinsically flexible loop and the flexibility of this loop changes upon ligand binding, suggesting the importance of sequence specific dynamics for Pin1 substrate (Namanja et al., 2007).

**Regulation of Pin1 function**

Pin1 function is tightly controlled at multiple levels under various physiological conditions. Substrate availability controls the subcellular localization and function of Pin1 due to presence of Ser/Thr-Pro motifs (Lu et al., 2002). Transcription factor E2F controls the expression of Pin1 in response to growth factors (Ryo et al., 2002). Tumor suppressor breast cancer-1, early onset (BRCA-1) is one of the most prominent gene that suppresses the expression of Pin1 (MacLachlan and El-Deiry, 2000). Pin1 is also regulated by various post-
translational modifications. Phosphorylation of Pin1 is regulated in a cell cycle dependent manner which includes phosphorylation on Ser 16 in the WW domain, resulting in blocking of Pin1 interaction with its substrates (Lu et al., 2002). Additionally, Polo-like kinase-1 (PLK1) also phosphorlylates Pin1 on Ser65, resulting in increased stability of Pin1 by reducing its ubiquitination (Eckerdt et al., 2005). Moreover, Pin1 enzymatic activity is blocked by oxidative modifications that occur in the early stages of Alzheimer’s disease (Sultana et al., 2006).

**Role of Pin1 in controlling biological activities**

Pin1 interacts with and regulates the stability of its phospho-substrates, at the post-phosphorylation levels, thereby controlling their biological processes. Numerous studies have shown the Pin1 catalyzed conformational regulation has a profound impact on the regulation of cell cycle proteins, growth, stress responses, immune responses, germ cell development, neuronal differentiation and survival (Atchison and Means, 2004; Braithwaite et al., 2006). Deregulation of Pin1 signaling is implicated in a growing number of pathological conditions such as Alzheimer’s disease (AD) (Akiyama et al., 2005; Maruszak et al., 2009), asthma (Esnault et al., 2008), frontotemporal dementia (Thorpe et al., 2004), Parkinsonism linked to chromosome 17 (FTDP-17) (Yotsumoto et al., 2009), Pick disease (Ramakrishnan et al., 2003), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD) (Lee et al., 2001) and cancer (Li et al., 2006).

**Pin1 in controlling the stability of cell cycle proteins in cancers**

In many human cancers, Pin1 protein is overexpressed and this overexpression is associated with poor clinical outcome. Pin1 can promote oncogenesis by upregulating the expression of oncoproteins and disrupting cell cycle progression. Pin1 regulates the
expression of cyclinD1 by controlling the Ras signaling, resulting in inhibition of β-catenin and the tumor suppressor APC (Ryo et al., 2002). Occurrence of overexpressed cyclinD has been found in about half of breast cancer patients (Bartkova et al., 1996). In cell culture studies, Pin1 regulates the stability and subcellular localization of β-catenin. Its increased expression also correlates with upregulated Pin1 expression in breast tumors (Ryo et al., 2001). Pin1 also binds and isomerizes the phosphorylated Ser246-Pro motif in β-catenin and thus inhibits the interaction of β-catenin with APC, which plays role in exporting nuclear β-catenin into the cytoplasm for degradation. Pin1 further binds to cyclin D1 at the phosphorylated Thr-286-Pro motif and catalyses its isomerization which results in stabilization and accumulation of cyclin D1 in the nucleus (Liou et al., 2002). After Pro-directed phosphorylation by JNK, the activity of c-Jun is also controlled by Pin1. Pin1 increases the protein stability of c-Jun by inhibiting c-Jun polyubiquitination (Wulf et al., 2002). Pin1 also positively correlates the expression of the anti-apoptotic protein, myeloid cell leukemia (MCL-1) whose elevated expression leads to poor survival rate of breast cancer patients (Ding et al., 2008). Additionally, Pin1 is identified as the novel regulator of extracellular signal-regulated kinase (ERK) (Ding et al., 2008). In addition to stabilizing and activating oncoproteins, Pin1 can also inactivate and destabilizes a huge number of tumor suppressors and cell growth inhibitors. Pin1 specifically isomerizes the cis/trans conformation of phosphorylated Thr58-Pro of c-Myc, which in turn enhances the dephosphorylation activity of protein phosphatase 2A (PP2A) on Ser62. Similar to c-Myc, misregulation of cyclin E function also causes cell cycle defects and onset of oncogenesis (Yeh and Means, 2007). Pin1 facilitates Cyclin E protein degradation during cell cycle progression, whereas, the absence of Pin1 results in an accumulation of cyclin E at the G1/S phase of the cell cycle
Pin1 also regulates a variety of oncoproteins associated with transcription by increasing β-catenin and nuclear factor kappa b (NF-κB) stability, and decreasing the stability of c-Myv, cyclin E and RAR. Additionally, Pin1 also controls other transcription-associated proteins such as steroid receptor coactivator-3 (SRC-3) that is overexpressed in breast and ovarian cancers (Yi et al., 2005). Pin1 regulates transforming growth factor-beta (TGF-β) signaling which is essential in regulating proliferation, differentiation, migration and apoptosis (Siegel and Massague, 2003). Additionally, it has been found that Pin1 binds to phosphorylated Ser670 of GRK2 (G protein-coupled receptor kinase 2, plays role in cancer formation) and triggers GRK2 degradation (Nigg, 2001).

**Pin1 in neuronal function and apoptosis**

Pin1 is expressed in most neurons at high levels (Ryo et al., 2001; Wulf et al., 2001). Also, Pin1 expression is induced during differentiation process of neurons (Hamdane et al., 2006). Pin1 regulates several neuronal proteins that are important for neuronal function and survival including tau, amyloid precursor protein (APP), and myeloid cell leukemia sequence (MCL-1) (Zhou et al., 2000; Pastorino et al., 2006; Li et al., 2007). High expression levels of Pin1 in terminally differentiated and post mitotic neurons suggest that it plays several important roles in neurons except cell cycle regulation and proliferation (Becker and Bonni, 2006). Pin1 interacts with Ser65 phosphorylated BIMEL and thereby stabilizes BIMEL, resulting in neuronal apoptosis (Becker and Bonni, 2007). All survival and death signaling pathways in neurons converge at the mitochondria where they integrated mainly by the Bcl2 family of proteins. The proapoptotic ‘BH3 only’ subfamily is the only members critical for the activation of mitochondria mediated cell death as they are upstream molecules of the apoptotic pathway. JNK signaling is believed as one of the key regulator of BIMEL in
neurons (Becker and Bonni, 2007). The selective enrichment of Pin1 at the mitochondrial membrane of BIMEL occurs through binding of WW domain of Pin1 to Ser65-phosphorylated site of BIMEL which contains JNK interacting protein 3 (JIP3). Pin1 associates with JIP3 in neurons in a phosphorylation dependent manner (Becker and Bonni, 2007). In addition to upregulating BIM gene expression through the activation of c-JUN, JNK directly phosphorylates the BIMEL protein at serine 65 which leads to activation of apoptotic function of BIMEL in neurons, but suppresses cell death in proliferating non-neuronal cells, possibly through the proteosomal degradation of phosphorylated BIMEL. In addition to that, Pin1 induced apoptotic death in NGF-treated PC12 neuronal cells is blocked by expression of dominant negative-Jun (Barone et al., 2008). In contrast, Pin1 knockout mice show increased oligodendrocytes apoptosis after spinal cord injury (Li et al., 2007) and untreated aged Pin1 (-/-) mice show patterns of neurodegeneration features, suggesting pro-survival role of Pin1.

**Pin1 and Alzheimers disease**

Although most human neurons express Pin1, its levels are abnormally low in the Alzheimer’s disease (AD) brain. The neuropathological hallmarks of AD include presence of neurofibrillary tangles and neurite plaques in the brain. The neurofibrillary tangles consist of paired helical filaments composed of hyperphosphorylated microtubule-associated protein tau. Similarly, the neuritic plaques appear due to over production or lack of degradation of amyloid-b (Ab) peptides derived from APP (Amtul et al., 2002; Mattson and Chan, 2003). Pin1 promotes dephosphorylation of the pThr231-Pro motif in tau by the trans-specific protein phosphatase PP2A (Lu et al., 1999). Pin1 knockout promotes accumulation of the pThr231-Promotif in the tangle-specific conformation (Zhou et al., 2000). So, Pin1 promotes
tau dephosphorylation and restore tau function possibly by catalyzing cis to trans isomerization. So in absence or reduced activity of Pin1, the cis pThr231-Pro motif accumulates, leading to tau hyperphosphorylation, aggregation and tangle formation. In addition to that Pin1 also promotes APP processing and Ab production (Amtul et al., 2002). Pin1 interacts with pThr668-Pro motif of APP and causes its isomerization. In the mouse, it has been reported that the absence of Pin1 promotes amyloidogenic APP processing and increases the toxic substance Aβ42 in an age dependent manner (Pastorino et al., 2006). Collectively, Pin1 plays pivotal role in protecting against age-dependent neurodegeneration in AD and might be a new drug target for treating the disease.

**Pin1 and Parkinson’s disease**

Pin1’s level, activity and function in the pathogenesis of PD are completely unknown. Recently, Ryo et al., 2006 reported that Pin1 accumulates in lewy bodies (which is a cytoplasmic inclusions containing α-synuclein), and promotes the formation of α-synuclein inclusions by protecting α-synuclein from degradation in kidney cells (Ryo et al., 2006). But, α-synuclein degradation is not directly linked to Pin1 PPIase activity, suggesting that Pin1 and α-synuclein do not have direct interaction. However, Pin1 binds sunphilin-1, an alpha-synuclein regulatory protein at pSer211-Pro and Ser215-Pro motifs and enhances its interaction with α-synuclein, facilitating the formation of α-synuclein inclusions (Ryo et al., 2006).

**Pin1 and immune responses**

The fundamental effect of Pin1 on protein conformation accounts for its diverse role in cell cycle progression, proliferation and transcription. Pin1 also increases the activity of nuclear factor kappa beta (NF-κB), a transcription factor that plays a crucial role in
inflammation. Pin1 also inhibits degradation of β-catenin and NF-κB p65, increasing their levels (Monje et al., 2005). All these transcription factors are associated with induction of inflammatory gene products. In response to cytokine signals, Pin1 binds to and isomerizes the phosphorylated p65 subunit of NF-κB and prevents NF-κB from binding to IκB (inhibitor of NF-κB), resulting in increased NF-κB activity (Ryo et al., 2003). Pin1 has been reported to inhibit ubiquitin mediated proteolysis of p65 by SOCS1 (suppressor of cytokine signaling-1) (Ryo et al., 2003). Additionally, Pin1-deficient mice and cells are resistant to NF-κB mediated cytokine upregulation. Pin1 has also been shown to activate the transcription factor activating protein-1 (AP1) (Monje et al., 2005). Inducible nitric oxide synthase (iNOS) is one of the inflammatory genes activated by NF-κB and AP-1 (Xie et al., 1993; Du et al., 2006). Pin1 may also contribute to the onset of allergic asthma and microbial infection (Shen et al., 2005; Saitoh et al., 2006). In addition to that, Pin1 interacts with phosphorylated IRF3 and promotes its polyubiquitination and proteasome-dependent degradation thus facilitates innate immunity against viral infection (Saitoh et al., 2006).

Pin1’s roles during apoptosis are varied. While inhibition of Pin1 led to apoptosis in vascular smooth muscle cells and Pin1 was required for pro-survival signaling in eosinophils (Shen et al., 2009), Pin1’s pro-apoptotic function has been demonstrated during downregulation inhibitor of apoptosis protein-survivin in neuroblastoma cells (Dourlen et al., 2007) and promoting tumor cell death by dissociating tumor suppressor p53 from apoptosis inhibitor iASPP (Mantovani et al., 2007). In the nervous system, Pin1 has been shown to regulate oligodendrocyte apoptosis after spinal injury by binding and stabilizing Mcl-1 after JNK3 activation (Li et al., 2006) and also induce death of developing neurons during JNK signaling.
<table>
<thead>
<tr>
<th>Substrate name</th>
<th>Pin1 binding motif</th>
<th>Role of Pin1</th>
<th>Pathways/disease involved</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synphilin-1</td>
<td>Ser211, Ser215</td>
<td>Promotes lewy body formation; stabilizes α-synuclein</td>
<td>Parkinson’s disease</td>
<td>(Ryo et al., 2006)</td>
</tr>
<tr>
<td>c-Fos</td>
<td>C-terminal</td>
<td>Regulates stability and transcriptional activities</td>
<td></td>
<td>(Monji et al., 2005)</td>
</tr>
<tr>
<td>c-Jun</td>
<td>Ser63, Ser73</td>
<td>Regulates transcriptional activities</td>
<td>Breast cancer</td>
<td>(Wulf et al., 2001)</td>
</tr>
<tr>
<td>β-Catenin</td>
<td>Ser246</td>
<td>Regulates stabilization, localization and activation</td>
<td>Cell proliferation; Cancers</td>
<td>(Ryo et al., 2001)</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>Thr286</td>
<td>Regulates stabilization, localization and activation</td>
<td>Cell proliferation; Cancers</td>
<td>(Liou et al., 2002)</td>
</tr>
<tr>
<td>NF-κB/p65</td>
<td>Thr254</td>
<td>Neuclear translocation and stabilization</td>
<td>NF-κB activation; Cytokine release</td>
<td>(Ryo et al., 2003)</td>
</tr>
<tr>
<td>Akt</td>
<td>Thr92, Thr450</td>
<td>Regulates stability</td>
<td>Oncogenin pathways</td>
<td>(Liao et al., 2009)</td>
</tr>
<tr>
<td>P53</td>
<td>Ser33, Thr181, Ser315</td>
<td>Stabilization and promoter binding activities</td>
<td>DNA damage; Genotoxic stress</td>
<td>(Wulf et al., 2002; Cohen et al., 2008)</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Not known yet</td>
<td>Increases the bioactivity, prevents, dephosphorylation</td>
<td>Apoptotic pathways</td>
<td>(Basu et al., 2002)</td>
</tr>
<tr>
<td>BIMEL</td>
<td>Ser65</td>
<td>Stabilization and apoptosis</td>
<td>Neuronal apoptosis</td>
<td>(Becker and Bonni, 2006)</td>
</tr>
<tr>
<td>SMRT</td>
<td>Ser1241, Thr1445</td>
<td>Destabilization</td>
<td>Lymphoma; Leukemia; Prostate cancer</td>
<td>(Stanya et al., 2008)</td>
</tr>
<tr>
<td>Oct4</td>
<td>Ser12</td>
<td>Modulates stability and transcriptional activity</td>
<td>Stem cell pluripotency</td>
<td>(Nishi et al., 2011)</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>Ser84</td>
<td>Prevents polyubiquitination</td>
<td>Atherosclerosis</td>
<td>(Fujimoto et al., 2010)</td>
</tr>
<tr>
<td>c-Myc</td>
<td>Thr58</td>
<td>Degradation</td>
<td>Tumorigenesis</td>
<td>(Yeh et al., 2004)</td>
</tr>
<tr>
<td>Cyclin E</td>
<td>Ser384</td>
<td>Regulation of cellular levels</td>
<td>Cell cycle; Tumorigenesis</td>
<td>(Yeh et al., 2006)</td>
</tr>
<tr>
<td>Tau</td>
<td>Thr231</td>
<td>Regulate protein stability and dephosphorylation</td>
<td>Taupathy; Neuronal differentiation; Alzheimer’s disease</td>
<td>(Lu et al., 1999; Lim et al., 2008)</td>
</tr>
<tr>
<td>Pim-1 kinase</td>
<td>Not known yet</td>
<td>Destabilization of Pim1</td>
<td>Lymphoma; Prostate cancer</td>
<td>(Ma et al., 2007)</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Not known yet</td>
<td>Regulates accumulation and translation in neutrophil</td>
<td>Chronic asthma</td>
<td>(Shen et al., 2008)</td>
</tr>
</tbody>
</table>
**Pin1 inhibitors**

Until now, there are few compounds that have been reported to act as inhibitors of Pin1. Among several inhibitors, Juglone is regarded as one of the most potent inhibitor of Pin1. Juglone is an aromatic organic naphthoquinone (5-hydroxy-1, 4-naphthalenedione, (C10H6O3), naturally occurs in the leaves, roots, and bark of plants in the black walnut. Naphthoquinone is known to have various physiological activities and also it plays role in induction of apoptosis (Kang et al., 2002).

Juglone irreversibly and specifically inactivates enzymatic activity of E. coli parvulin and human Pin1 by modification of thiol groups of cysteine residue in parvulin (Hennig et al., 1998). The inhibition of isomerase activity by juglone is caused by partial unfolding of the PPIase activity site. Also, juglone inhibits numerous parvulins as well as other enzymes comprising Cys residues in their catalytic domains.

![Figure 6. Structure of Juglone](image)

Juglone plays role in various cellular events such as induction of DNA damage (Wang et al., 2001a) and cell death (Paulsen and Ljungman, 2005). Recently, Esnault et al., 2007 (Esnault et al., 2008) showed that Juglone prevented acute and chronic rejection of MHC mismatched, orthotopic rat lung transplants by reducing the expression of IFN-γ and CXCL-10 mRNA stability, accumulation and protein expression after cell activation. Juglone also attenuates rheumatoid arthritis development and COX-2 expression in human primary culture RA chondrocytes and CII treated DBA/1J mice (Jeong et al., 2009). Additionally, recent reports suggested anti-inflammatory, anti-viral and anti-
fungal properties of Juglone (Omar et al., 2000). Juglone at a dose of 5.7μM in vitro blocked Pin1’s and E. coli parvulin isomerase activity completely and these doses of Juglone had no effect on other PPIases (Hennig et al., 1998). Besides juglone there are some other synthetic inhibitor of Pin1 such as PiB (diethyl-1,3,6,8-tetrahydro-1,3,6,8-tetraoxobenzo phenanthroline-2,7-diacetate) and PiJ (diethyl-1,3,8,10-tetrahydro-1,3,8,10-tetraoxoanthra diisoquinoline-2, 9-diacetate). The structures of PiB and PiJ have double-ring structures like juglone, but otherwise their overall structures are completely different from the juglone. Recently, a novel Pin1 inhibitor, dipenramethyle thiram monosulfide (DTM) has been identified (Tatara et al., 2009). DTM is soluble in DMSO, and is a competitive inhibitor of Pin1. Molecular modeling studies showed that DTM binds to the active site of PPIase domain of Pin1. In mouse embryo fibroblasts (MEF) lysate, DTM inhibited Pin1 PPIase activity but not FKBP or cyclophilin PPIases (Tatara et al., 2009). Additionally, several peptide mimetics have also been reported to inhibit Pin1 (Zhang et al., 2007b). Molecular Pin1 short interfering RNA (Pin1 siRNA) and dominant negative constructs of Pin1 also specifically inhibit the levels and activity of Pin1 respectively in situ.

**NF-κB**

The nuclear factor kappa B (NF-κB) is a key mediator of inducible transcription in the immune system. It consists of five related transcription factors that regulate inducible gene expression in various physiological processes. It was first identified in mature B and plasma cells by Sen and Baltimore (Sen and Baltimore, 1986). The mammalian NF-κB family consists of p65 (Rel A), RelB, c-Rel, 105/p50 (NF-κB1) and p100/p52 (NF-κB2) that associate with each other to form distinct transcriptionally active homo and heterodimer
complexes. They all share a conserved 300-amino acid-long amino-terminal REL homology domain (RHD) (Ghosh et al., 1998) and are regulated by eight IκB family members.

The amino terminal part of the RHD mediates specific DNA binding to NF-κB consensus sequences present in regulatory elements of NF-κB target genes (5′-GGGGPuNNPyPyCC-3′), whereas the carboxy-terminal part of the RHD is mainly responsible for dimerization and interaction with IκBs (Ghosh et al., 1995; Chen et al., 1998).

Role of NF-κB in neurodegenerative disorders

The nuclear factor kappa B or NF-κB refers to the Rel family of transcription factors consisting of five family members: p65/RelA, ReB, c-Rel, p50/p105 (NF-κB 1), and p52/p100 (NF-κB 2). The subunits exist in unstimulated cells as homo or heterodimers bound to inhibitor kappa
NF-κB was discovered in 1986 as the proinflammatory factor that responds to LPS signaling (Sen and Baltimore, 1986). Initially, NF-κB has been considered as an immunologic transcription factor. However, more recently, NF-κB activity has been implicated in diverse disease pathogenesis and biologic processes (Verma, 2004). NF-κB proteins are characterized by the presence of a conserved 300 amino acid Rel homology domain (RHD) that is located at the N terminus of the protein and is responsible for dimerization, interaction with IkB and binding to DNA. Additionally, there are two subgroups of the NF-κB family, three of the subunits, p65, relB and c-rel, contain transcription activation domain (TAD) at the c-terminus. The p50 and p52 subfamily members are proteolytically cleaved from larger proteins p105 and p100, respectively, and do not contain TADs. Instead they act as transcriptional suppressor. The p65/p50 heterodimer, the prototypical NF-κB dimer leads to the transcription of various genes, whereas the p50/p50 homodimer, which also binds to the NF-κB consensus sequence with a higher affinity than p65/p50, inhibits transcription (Adib-Conquy et al., 2000). NF-κB is activated through two different pathways: the canonical/activated and non-canonical pathways. The canonical pathway is generally induced by TNF-α, IL-1, TLR or LPS and uses a large variety of signaling adaptors to engage IKK activity. The non-canonical pathway depends on NIK (NF-κB -inducing kinase) induced activation of IKKa. The inhibitor kappa B kinase or IKK complex, which phosphorylates IkB following its activation, consists of two homologous catalytic kinase subunits, IKK-a and IKK-b, and the regulatory subunit IKKy or NEMO (NF-κB essential modifier) (May et al., 2000). Various molecules upstream to IKK are involved in the NF-κB signaling pathways. LPS activated TLR4 binds to MyD88 and IRAK, which then signal through TRAF6, leading to the activation of IKK. Other signaling
proteins include MyD88, RIP, NIK and TRAF (Hayden and Ghosh, 2004). IKK-g interacts with the upstream molecules, which results in the oligomerization of IKK-g. IKK-g then induces phosphorylation or possible autophosphorylation of the IKKa or b subunits. The catalytic subunits then release from IKK-g and phosphorylate IkB at Ser32 and Ser36. After phosphorylation, IkBa is poly-ubiquitinated at Lys22 and degraded by the 26S proteosome. This degradation mechanism leads to the loss of nuclear export signal provided by the IKB and activates the nuclear localization (NLS) signals on the NF-κB subunits. NF-κB then migrates to the nucleus where it binds to the promoter region of its target molecules and facilitates gene expression of pro-inflammatory, cell growth and other regulated genes (Tak and Firestein, 2001; Hayden et al., 2006; Hayden and Ghosh, 2008).

IkB protein family comprises three functional groups: the typical IkB proteins IkBα, IkBβ and IkBε, which are present in the cytoplasm of unstimulated cells and undergo stimulus-induced degradation and resynthesis. IkBα is a prototypical member of IkB family of proteins. As in other organ systems, functional NF-κB complexes are present in essentially all cell types in the nervous system, including neurons, astrocytes, microglia, and oligodendrocytes (O'Neill and Kaltschmidt, 1997). The most common subunits expressed are p50, p65, and IkBα. Receptor-linked signal transduction pathways that ultimately result in NF-κB activation, such as those activated by TNF-α and Fas ligand, have also been documented in neurons and glial cells (Bruce et al., 1996). Additional neuron-specific signals that activate NF-κB include nerve growth factor (NGF) (Carter et al., 1996) and the secreted form of β-amyloid precursor protein (βAPP) that is involved in Alzheimer’s disease (Mattson and Chan, 2003). As in other organs, NF-κB influences the expression of a complex array of genes in the nervous system and, in general, the genes serve important functions in cellular
responses to injury and in neuronal plasticity. Genes encoding of several different injury-responsive cytokines are induced by NF-κB in glia and neurons. These include TNF-α and IL-6, which are produced in particularly high amounts by microglia and astrocytes; βAPP, an injury-responsive cytokine/neurotrophic factor; the calcium-binding protein calbindin-D28k, which may play roles in modulating calcium-mediated neuronal signaling and cell death; inhibitor-of-apoptosis proteins (IAPs), which can protect neurons against apoptosis in experimental models of stroke and seizures; manganese superoxide dismutase (Mn-SOD), a mitochondrial antioxidant enzyme that has been shown to be neuroprotective; and Bcl-2, the prototypical member of the Bcl-2 family of antiapoptotic proteins. Additional genes induced by NF-κB in glial cells include the cell adhesion molecule intercellular adhesion molecule-1, the inducible form of nitric oxide synthase (iNOS), and glial fibrillary acidic protein (GFAP). Presence of consensus DNA binding sites for NF-κB in promoter regions of most proinflammatory molecules, and inhibition of induction of their expression upon inhibition of NF-κB activation, suggest that activation of NF-κB is important for the induction of proinflammatory molecules (Ghosh, 1999). NF-κB is activated by pro-inflammatory cytokines, induced cell proliferation and anti-apoptotic gene expression, and it also enhances angiogenesis via vascular endothelial growth factor expression (Aggarwal et al., 2004; Greten and Karin, 2004). Activation of NF-κB has been reported in various chronic inflammatory diseases including asthma, rheumatoid arthritis and EAE model of multiple sclerosis (Dasgupta et al., 2004). Postmortem analysis of human PD brain provide evidence of the activation of NF-κB in dopaminergic neurons of human PD patients (Hunot et al., 1997). Recently, Ghosh et al., 2007 demonstrated that NF-κB is required for the transcription of most of the proinflammatory molecules and it is activated in the SNpc of PD patients and
MPTP-intoxicated mice. Selective inhibition of NF-κB in mice by NBD (NEMO binding domain) peptides protected dopaminergic neurons from MPTP toxicity (Ghosh et al., 2007). Furthermore, other neurotoxins such as rotenone and 6-hydroxydopamine (6-OHDA) used in experimental models of PD, stimulate NF-κB (Blum et al., 2001; Wang et al., 2002; Ghribi et al., 2003). Based on these findings, it is plausible that the activation of NF-κB may be essential for neurotoxin-induced degeneration in nigral neurons.
Background and Literature Review II

In this section, the activation of the glial cells in PD and MPTP mouse model of PD, glial cells mediated inflammation and oxidative stress, different neuroprotective strategies to cure PD and mitochondria targeted neuroprotective compounds in mouse model of PD are reviewed.

Pathophysiology of PD

Despite intense research, the cause of dopaminergic neurodegeneration in PD is not known completely. Among different cellular and molecular changes that are believed to be involved in the progressive degeneration of dopaminergic neurons, neuroinflammatory mechanism is one of them (Dauer and Przedborski, 2003). Neuroinflammation mechanism comprises microglial and astroglial activation, proinflammatory cytokines and molecules and oxidative stress. It is now well documented that microglial activation leads to loss of DA neurons in PD patients. Levels of nitrite (NO$_2$), a metabolite of NO and inducible nitric oxide synthase (iNOS) are higher in the central nervous system (CNS) of human PD cases and in animal models of PD (Mogi et al., 1994). Consistent with this finding, iNOS knockout animals were resistant to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) -induced dopaminergic neuronal loss in the substantia nigra (Przedborski and Vila, 2003). One of the major sources of reactive oxygen species (ROS) in neurodegeneration is NADPH oxidase, a multimeric enzyme that generates both O$_2^-$ and H$_2$O$_2$ (Qureshi et al., 1995). In addition to that levels of proinflammatory cytokines (TNF-α, IL-1β, IFN-γ, IL-6), chemokines and prostaglandins levels are also elevated in PD brains (McGeer et al., 1988; Nagatsu et al., 2000). Recent reports suggest that NF-κB, a transcription factor that is required for transcription of proinflammatory molecules is also activated in the substantia nigra of PD
patients and MPTP-treated mice (Ghosh et al., 2007). Additionally, Nurr1, a nuclear orphan receptor suppresses glial activation by docking to NF-κB-p65 on target inflammatory gene promoters and protects against dopaminergic neurodegeneration in substantia nigra.

**Microglia and its activation**

Microglia constitutes about 10% of all glial cells and they are distributed all over the brain with maximum population in substantia nigra. Microglia was first identified by Del Rio Hortega in 1919 as mesodermal cells entering the brain in late embryonic phase (Hortega, 1919). Under normal condition, microglia displays a ramified morphology and is referred as resting microglia. In resting condition, microglia play a homeostatic role in the CNS respond to environmental stresses and immunological challenges by scavenging excess neurotoxins and removing dying cells and cellular debris (Carson et al., 2007; Tansey et al., 2008). It has been shown that early microglial activation within 24h of inflammatory stimulus exposure, leads to elevated microglial immunoglobulin reactivity, upregulation of cell adhesion molecule such as CD11b, CD18 and intracellular adhesion molecule (ICAM-1) (Orr et al., 2002). Furthermore, upon activation, the morphology of microglial cell changes. The cell body becomes enlarged, amoeboid shape with thickened processes (Kloss et al., 1999). In the activated state, microglia constitutively expresses MHC-II molecule, b2 integrin CD11a, CD11b, CD11c (Marlin and Springer, 1987; Greenwood et al., 2003). Stimuli that can trigger microglial

![Figure 8. Expression of CD11b-positive microglia in SN of Human brain](image)
activation include pathogenically modified CNS protein, antigens from infectious agents (such as gram-negative bacterial cell wall component liposaccharide (LPS)), prion proteins, α-synuclein, ATP, cAMP, and interleukin (IL)-1β, IL-6, and IL-10 (Hanisch, 2002; Nakamura et al., 2002). In addition to these, aging and repeated exposure to environmental toxins (including pesticides and particulate matter) may contribute to the increased permeability to the blood brain barrier which facilitates entry of peripheral immune cells into the brain, resulting in microgliosis. At the molecular level, microglial activation may be triggered by protein aggregation and formation of inclusions arising from mutation, disruption of ubiquitin-proteasome system, bacterial or viral infections or traumatic brain injury (Zhang et al., 2005; Austin et al., 2006; Kim et al., 2006).

Astrocytes - pro-inflammatory or anti-inflammatory

The function of astrocytes is not clear in inflammatory reactions. After injury, astrocytes migrate to the site of injury and develop a hypertrophic morphology with increased cell bodies and thickened processes and are known as reactive astrocytes. Astrocytes do not attack any microglia, instead astrocytes produce factors that are important in the inflammatory reactions, seen in SN in PD brains (Miklossy et al., 2006). Recent studies have demonstrated activated astroglial cells in human PD brain and in the MPTP mouse model of PD (Ghosh et al., 2007; Ghosh et al., 2009). Many ICAM-1 positive

Figure 9. Expression of GFAP-positive astrocytes in SN of Human brain
Astrocytes are also present in the SN in PD and this may attract reactive microglia to the area since such microglia carry counter receptor LFA-1 (Miklossy et al., 2006). A-synuclein has been shown to activate microglia and astrocytes to produce IL-6 and ICAM-1 (Klegeris et al., 2007). Astrocytes on the other hand, secrete many neurotrophic factors for dopaminergic neurons including glial cell derived neurotrophic factor (GDNF), brain derived neurotropic factor (BDNF) and mesencephalic astrocytes-derived factor (MANF) (Lin et al., 1993; Petrova et al., 2003; Chen et al., 2006; Saavedra et al., 2006). Astrocytes in the SN also upregulate protease-activated receptor-1 (PAR-1) in PD and this has a protective effect by increasing the activity of glutathione peroxidase (Ishida et al., 2006). Recently, it has been shown that astrocytes express NF-E2-related factors (Nrf2), which binds to the antioxidant response element to induce antioxidant enzymes (Jakel et al., 2007). Moreover, overexpression of Nrf2 in astrocytes is reported to protect against 6-OHDA damage in mice (Jakel et al., 2007). Based on all this evidence, it is very difficult to conclude whether astrocytes are pro-inflammatory or anti-inflammatory.

**Glial activation in MPTP mouse model of PD**

A glial activation including microglia and astrocytes has been widely described in several animal models of PD. Herein, glial activation in MPTP mouse model of PD is discussed. In mice and monkeys, MPTP-induced activation of microglia has been reported by several research groups (Hurley et al., 2003; McGeer et al., 2003; Barcia et al., 2004). However, the method of MPTP injection (dosing regimen) plays important role in the activation of microglia. In acute MPTP model of PD, robust activation of microglial cells have been observed whereas, in sub-acute MPTP model, comparatively less microglial activation occurred (Barcia et al., 2004; Furuya et al., 2004; Ghosh et al., 2009). Microglial
activation is also seen after osmotic infusion of complex-I inhibitors in rats (Sherer et al., 2003; Champy et al., 2004). Moreover, the direct role of the microglial activation in the events leading to neuronal degeneration is supported by animal studies showing that microglial activation precedes neuronal degeneration (Liberatore et al., 1999).

Figure 10. Cellular events in substantia nigra after MPTP administration (Hirsch et al., 2009).

An astroglial reaction has been reported in the substantia nigra and striatum of the mice exposed to MPTP (Czlonkowska et al., 2001; Breidert et al., 2002). Astroglial reaction in acute MPTP model generally starts from 24h after the last dose of MPTP injection and at 48h after MPTP (Breidert et al., 2002). In terms of cell proliferation, the astroglial and microglial reactions also differ. Newborn microglial cells have been demonstrated by BrdU labeling in mice and monkeys exposed to MPTP, whereas, no such staining was observed for GFAP positive astroglial cells (Brochard et al., 2009). Microgliosis is involved with an increased amount of microglial cells and morphological change of microglial cells, whereas astrogliosis is involved with increased expression and morphological changes of the astrocytes, but no increase in cell number.

Neuroinflammatory mechanism
The involvement of inflammation in PD was first reported by McGeer et al., 1988, where they observed the upregulation of major histocompatibility complex (MHC) in PD patients (McGeer et al., 1988). Similarly, increased levels of b2-microglobulins in the striatum of PD patients were reported (Mogi et al., 1995). Subsequently, a wide range of other proinflammatory molecules has been implicated in the process (Arai et al., 2004). Activated microglial cells and reactive astrocytes are the major modulators of inflammatory reaction in the brain. After activation of the glial cells, they are capable of producing toxicity through the production and release of oxygen- and nitrogen-derived products in a process known as respiratory or oxidative bursts. This process of toxicity is based on the regulated induction of NADPH (nicotinamide adenine dinucleotide phosphate) oxidase, iNOS (inducible nitric oxide synthase) and MPO (myeloperoxidase). NADPH oxidase is the source of superoxide, whereas, iNOS produces nitric oxide (NO) and MPO regulates production of hypochlorous acid (HOCl).

**Table 2. Evidence of Neuroinflammation in PD brain**

<table>
<thead>
<tr>
<th>Human/ Model systems</th>
<th>Evidences</th>
<th>References</th>
</tr>
</thead>
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<tr>
<td>Human PD brain</td>
<td>Higher no. of activated microglia (HLA-DP, HLA-DQ, HLA-DR and ferritin) in SN</td>
<td>(McGeer et al., 1988)</td>
</tr>
<tr>
<td>MPTP model</td>
<td>Microglia activation in close proximity to dopaminergic neurons. Sustained microglial activation 1 year after MPTP injection</td>
<td>(Gao et al., 2003; Barcia et al., 2004)</td>
</tr>
<tr>
<td>Human PD brain</td>
<td>Astrogliosis evidenced by increased GFAP and glutathione peroxidase immunostaining</td>
<td>(Damier et al., 1993)</td>
</tr>
<tr>
<td>MPTP model</td>
<td>Astroglial activation starts 24 h after MPTP treatment</td>
<td>(Breidert et al., 2002)</td>
</tr>
<tr>
<td>Human PD brain</td>
<td>Increased levels of proinflammatory cytokines (IL-1β, TNF-α, IFN-γ, CD23) in the CSF and striatum</td>
<td>(Mogi et al., 1994; Hunot et al., 1999)</td>
</tr>
<tr>
<td>MPTP model</td>
<td>Upregulation of iNOS in SN; iNOS knock out mice protective against MPTP toxicity</td>
<td>(Wu et al., 2002)</td>
</tr>
<tr>
<td>Human PD brain</td>
<td>Increased levels of iNOS and COX-2 in SN and striatum</td>
<td>(Hunot et al., 1996)</td>
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</table>
NADPH oxidase

NADPH oxidase (also known as PHOX) is a membrane-bound enzyme, which leads to the production of O$_2^-$ from oxygen in the microglial cells leading to damage to the dopaminergic neurons (Gao et al., 2003; Wu et al., 2005a). NADPH oxidase is a multimeric enzyme composed of plasma membrane bound gp91phox and p22phox subunits and cytosolic p40phox, p47phox and p67phox subunits. Upon activation, the cytosolic subunits undergo phosphorylation and translocate to the membrane, where together with small G proteins they associate with the membrane bound subunits. The assembled and active enzyme complex then catalyzes the transfer of a single electron from NADPH to molecular oxygen to release superoxide. In normal conditions, NADPH oxidase remains inactive in resting microglial cells, but is activated when the microglia becomes activated (Babior, 2000). By the stimulation of multiple proinflammatory stimulus, including environmental stimuli such as LPS, MPTP, nanoparticles, diesel exhaust particles, rotenone, paraquat and endogenous proteins such as α-synuclein, β-amyloid, microglia generates reactive oxygen species (ROS) (Block and Hong, 2005). In mice lacking a functional NADPH oxidase because of the deletion of gene for gp91phox subunit, significant protection against MPTP-induced loss of nigral DA neurons and degeneration of striatal dopaminergic fibers is observed (Wu et al., 2003; Zhang et al., 2004). LPS-mediated loss of nigral DA neurons in vivo and in vitro is significantly less in NADPH oxidase-deficient mice compared to NADPH oxidase (+/+) mice (Qin et al., 2004).
Nitric oxide (NO) is another potential source of oxidative stress and inflammation. NO is produced by nitric oxide synthase (NOS) by converting of L-arginine to L-citrulline utilizing NADPH oxidase and O₂ as cofactors (Duval et al., 1996; Day et al., 1999). There are 3 isoforms of NOS: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS), out of which inducible nNOS is expressed in several neuronal subtypes except dopaminergic neurons. In contrast to nNOS, iNOS is not normally expressed in the brain; however, in the pathological condition iNOS can be induced. Under many conditions, activated glial cells produce iNOS which leads to increased production of NO. CD23 mediated increase in iNOS has been reported in the SN of patients with PD (Hunot et al., 1996). MPTP administration to mice also produces glial cells mediated increase in iNOS expression and NO production (Liberatore et al., 1999). Consequently, mice laking iNOS gene is less sensitive to MPTP-induced loss of SN DA neurons (Itzhak et al., 1998). MPTP-induced striatal dopamine depletion is however not affected in iNOS null mice, nor was
MPTP induced microglial activation (Liberatore et al., 1999; Dehmer et al., 2000). Although poorly reactive, NO and $O_2^-$ free radicals can combine together to form the highly reactive nitrogen species peroxynitrite (ONOO$^-$), which can cause oxidative damage to various proteins such as tyrosine hydroxylase (TH) and α-synuclein (Ara et al., 1998; Przedborski and Vila, 2001). Iron content in SN is increased in PD patients and in animal models of the disease (Hirsch, 2006). Through a superoxide-driven Fenton’s reaction between hydrogen peroxide and the free ferrous iron catalyst, a substantial amount of highly reactive hydroxyl radicals (OH) might be produced. Reactive astrocytes produce myeloperoxidase (MPO) which oxidizes non-reactive nitrites (NO$_2^-$), the end product of free radical NO to reactive NO$_2^-$ free radicals which in turn also contribute to protein nitrosylation (van der Vliet et al., 1997). MPO, on other hand also implicated in production of non-radical oxidant hypochlorous (HOCl), which can damage macromolecules directly (Hampton et al., 1998). Altogether, an inflammatory, oxidative environment can be produced by activated glial cells in SN region.

**Proinflammatory cytokines**

Along with reactive oxygen species (ROS) and reactive nitrogen species (RNS), activated glial cells also produce proinflammatory molecules and cytokines and together form the basis of many neuroinflammatory responses (Noack et al., 1999; Heo et al., 2001). Increased proinflammatory cytokines such as IL-1β, IL-6, TNF-α and interferon γ (IFN-γ) are observed in the post-mortem brain as well as in cerebrospinal fluid and nigrostriatum of deceased PD patients (Mogi et al., 1994; Blum-Degen et al., 1995; Muller et al., 1998). In humans, the death signaling receptor TNF-α receptor-1 (TNF-R)-1, but not TNF-R2 has been found to be expressed in dopaminergic neurons in the SN and activation of TNF-R1 is toxic
to neurons (Mogi et al., 2000; Fontaine et al., 2002). Animal studies support an involvement of these proinflammatory cytokines in PD. A double knockout mice lacking both TNF receptors exhibit protection against MPTP toxicity (Sriram et al., 2002). Results from in vivo experiments suggest that IL-1β is toxic to neurons (Martin et al., 2002). Additionally, IL-6 knockout mice exposed to methamphetamine, show reduced neurodegeneration in SN (Ladenheim et al., 2000).

**Neuroprotective strategies**

As discussed previously in this thesis, PD is a multifactorial disease with oxidative stress, mitochondrial dysfunction and inflammation play key roles in dopaminergic neurodegeneration in nigrostriatum. For last few years, there have been increased efforts to search for neuroprotective agents that will protect the irreversible loss of neurons. In this part, the current evidence for prevention or slowing of the development of PD by the neuroprotective agents will be summarized.

**Anti-inflammatory agents**

As the evidence of detrimental role of inflammation in the pathogenesis of PD accumulate, a pool of anti-inflammatory agents is now under investigation. Data generated from non-steroidal anti-inflammatory drugs, microglial inhibitors and anti-inflammatory cytokines in cellular and animal studies have supported the notion that control of neuroinflammation and oxidative stress is a valuable strategy to retard or prevent dopaminergic neurodegeneration in the progression of PD.

*Non-steroidal anti-inflammatory agents (NSAIDS)*

Non-steroidal anti-inflammatory drugs such as aspirin, ibuprofen and indomethacin inhibit COX activity to block the production of proinflammatory lipid mediators,
prostaglandins. It has also been reported that NSAIDS lowers the risk of PD in epidemiological studies (Chen et al., 2003; Ton et al., 2006; Gao et al., 2011) and protects against neuronal death by scavenging reactive oxygen species (ROS) in neuronal cells (Grilli et al., 1996) inactivating the proinflammatory transcription factor NF-κB (Kopp and Ghosh, 1994) and activating the peroxisome proliferator-activated receptor-γ (PPARγ), a mediator of anti-inflammatory activities in microglia (Bernardo et al., 2005). Pretreatment of NSAIDS is neuroprotective in MPTP and 6-OHDA models of PD and in vivo (Esposito et al., 2007). Some reports suggest that ibuprofen reduces the risk of PD, but another NSAIDS, acetaminophen fails to attenuate the disease progression (Chen et al., 2005; Gao et al., 2011). A case-controlled study also demonstrates that NSAIDS and aspirin show no association with altering the risk of PD (Becker et al., 2011). In contrast, some studies also report that aspirin triggers increased risk of PD (Bower et al., 2006; Hernan et al., 2006). In contrast, other studies report that aspirin scavenges ROS and is neuroprotective (Di Matteo et al., 2006; Maharaj et al., 2006). In conclusion, NSAIDS could be valuable with a decreased risk of developing PD, but their efficacy in halting the disease progression is questionable.

**Minocycline**

Minocycline is a semi-synthetic second-generation lipophilic, tetracycline that crosses the blood brain barrier and exerts anti-inflammatory and antioxidant effects (Ryan et al., 2001; Faust et al., 2009). Minocycline inhibits the microglial activation and p38MAPK cascade which reduces release of proinflammatory cytokines and NO, thereby protecting neuronal death (Du et al., 2001; Tikka and Koistinaho, 2001; Wu et al., 2002). It also blocks the release of cytochrome c from mitochondria and expression of caspase-1 and caspase-3 (Chen et al., 2000; Zhu et al., 2002). In animal models of PD, minocycline treatment has
shown to be effective in MPTP-, 6-OHDA-, LPS-induced dopaminergic neurodegeneration (Du et al., 2001; He et al., 2001; Quintero et al., 2006).

Table 3. Anti-inflammatory agents in PD models

<table>
<thead>
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<th>Neuroprotective agents</th>
<th>Model systems</th>
<th>Results</th>
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<tr>
<td>Aspirin &amp; Ibuprofen</td>
<td>MPTP mouse; Human PD clinical trial</td>
<td>Restored neurotransmitter levels and protected dopaminergic neurons; lowered the risk of PD</td>
<td>(Chen et al., 2003; Chen et al., 2005)</td>
</tr>
<tr>
<td>Minocycline</td>
<td>MPTP mouse; Human clinical trial</td>
<td>Prevented MPTP-induced neurotransmitter loss in striatum and protected dopaminergic neurons; effective in slowing down the progressive nature of PD</td>
<td>(Tikka and Koistinaho, 2001; Wu et al., 2002; NINDS, 2006)</td>
</tr>
<tr>
<td>Rasagiline</td>
<td>MPTP and 6-OHDA mice model; Human PD clinical trial</td>
<td>Restored neurotransmitter levels and protected dopaminergic neurons; reduced long term progression of PD</td>
<td>(Heikkila et al., 1984; Hauser et al., 2009)</td>
</tr>
<tr>
<td>Estrogen</td>
<td>Human clinical trial</td>
<td>Reduced the symptoms in PD patients</td>
<td>(Shulman, 2007)</td>
</tr>
<tr>
<td>Nicotine</td>
<td>LPS and 6-OHDA mice models; Human clinical trial</td>
<td>Protected nigral dopaminergic neurons; restored striatal dopamine levels; lowered the risk of developing PD</td>
<td>(Johnston, 1976; Costa et al., 2001a)</td>
</tr>
<tr>
<td>Melatonin</td>
<td>MPTP and 6-OHDA rodents model; human clinical trial</td>
<td>Protected nigral dopaminergic neuronal loss; improved duration of sleep and reduced sleep disturbances</td>
<td>(Ferger et al., 1998; Costa et al., 2001b)</td>
</tr>
<tr>
<td>Phenybutyrate</td>
<td>MPTP and 6-OHDA model; No clinical trial yet</td>
<td>Protected dopaminergic neurons in SN; reduced impairment in motor and cognitive function in mice</td>
<td>(Gardian et al., 2004; Zhou et al., 2011)</td>
</tr>
<tr>
<td>Statins</td>
<td>MPTP and 6-OHDA model; No clinical trial yet</td>
<td>Protected dopaminergic neurons in SN; restored striatal dopamine; protected behavioral activities</td>
<td>(Ghosh et al., 2009; Ritz et al., 2010)</td>
</tr>
<tr>
<td>NBD peptide</td>
<td>MPTP model; No clinical trial yet</td>
<td>Protected dopaminergic neurons in SN; restored striatal dopamine; protected behavioral activities</td>
<td>(Ghosh et al., 2007)</td>
</tr>
<tr>
<td>Caffeine</td>
<td>MPTP and 6-OHDA models of PD; human clinical trial</td>
<td>Restored dopamine levels; decreased motor dysfunction; decreased risk of PD</td>
<td>(Costa et al., 2010; Kachroo et al., 2010)</td>
</tr>
<tr>
<td>GDNF</td>
<td>MPTP and 6-OHDA rodents and primates PD</td>
<td>Restored dopamine levels</td>
<td>(Wu et al., 2005b)</td>
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However, other studies have demonstrated that it exacerbates the MPTP-induced behavioral imparities, striatal terminal loss and damage to DA neurons *in vivo* and *in vitro* (Yang et al., 2003; Diguet et al., 2004). However, recent results from a Phase II clinical trial shows effectiveness of minocycline in slowing down the disease progression, without any adverse events in PD patients. Thus it suggests that minocycline should be considered for Phase III clinical trial (2008).

*Rasagiline*

Rasagiline (N-propargyl-1 (R)-aminoindam) is a highly selective and irreversible potent MAO-B inhibitor that attenuates MPTP and 6-OHDA-induced dopaminergic neuronal loss *in vivo* (Rabey et al., 2000; Youdim et al., 2001). Chronic administration of Rasagiline improves motor abnormalities and dopaminergic neuron survival in mice (Blandini et al., 2004). Rasagiline also increases the expression of different neurotrophins such as BDNF, GDNF and NGF (Maurer et al., 2003). By inhibiting caspase-3 and PARP-1 activation and translocation of GAPDH, Rasagiline suppresses the mitochondrial apoptosis (Akao et al., 2002; Weinreb et al., 2004). It also increases the expression of anti-apoptotic protein Bcl-2 through the PKC pathway (Akao et al., 2002). In humans Rasagiline successfully reduced the long term progression and symptoms of PD (Hauser et al., 2009; Olanow et al., 2009). Recently, in phase III clinical study, Rasagiline delayed the need for antiparkinsonian drugs and patients had lower scores on the PD symptoms rating scale (Rascol et al., 2011).

*Pioglitazone and rosiglitazone*

Pioglitazone and rosiglitazone are the agonists of the nuclear hormone receptor PPARg and currently approved for the treatment of type II diabetes. In the CNS, by inhibiting oxidative stress, inflammation and apoptosis, they exhibit neuroprotective effects
in models of neurodegenerative disorders (Chaturvedi and Beal, 2008). Recent study has demonstrated that by attenuating MAO-B, pioglitazone prevents MPTP-induced activation of nigral microglial cells and protects dopaminergic neurons in mice (Dehmer et al., 2004; Quinn et al., 2008). Recently, the neuroprotective effects of rosiglitazone have been shown in the MPTP model of PD (Schintu et al., 2009).

*Caffeine and nicotine*

Recent epidemiological studies support neuroprotective role of drinking caffeinated beverages (Ross et al., 2000; Saaksjarvi et al., 2008), although one study showed no protection (Xu et al., 2006). Chronic administration of caffeine protects against paraquat and maneb-induced neurotoxicity in mice (Kachroo et al., 2010). Additionally, in MPTP-treated mice, caffeine protects behavioral imparities and striatal dopaminergic terminals (Chen et al., 2001). Nicotine is comparable to caffeine with regards to lowering the risk of developing PD (Simon et al., 2009). In a recently published paper on Chinese population, there is a significant reduced rate of PD in individuals who drank coffee and smoked cigarettes (Tan et al., 2003). Additionally, in humans, acute nicotine treatment can improve impaired behavioral symptoms in PD patients (Holmes et al., 2011). Currently, nicotine is in phase II clinical trial focusing on optimizing dosage and increasing sample size.

*Others*

In addition to the above mentioned agents, there are several compounds that are also successful in different neurotoxins based animal models and clinical trials. Uric acid (UA), a natural antioxidant reduces the risk of PD in a dose dependent manner (de Lau et al., 2005). Vitamin E (a-tocopherol), lipid soluble anti-oxidant, has been shown to reduce oxidative stress caused by iron accumulation in the brain (Lan and Jiang, 1997), whereas, co-treatment
of vitamin E and vitamin C shows a decrease in PD progression in early stages of PD (Fahn, 1992). The role of vitamin D in PD is controversial. Some studies suggest its neuroprotective properties in 6-OHDA and cell culture models, where as in one case-controlled human study they found an increased risk of PD with high consumption of vitamin D (Anderson et al., 1999; Wang et al., 2001b). Additionally, various molecules including glial cell line-derived neurotrophic factor, neurturin, BDNF, TGF-β, and basic FGF showed some protective effects in animal models of PD (Flanders et al., 1998; Kordower et al., 2000). However, clinical application of those molecules has been limited because of difficulties in delivery and side effects. These peptides do not readily diffuse across the BBB or ventricular lining and have limited or unstable bioavailability and some toxicity (Kordower et al., 1999). However, Ghosh et al, (2007) have shown that NBD peptide, a selective NF-κB inhibitor and which has Antennapedia homeodomain that crosses the blood brain barrier of mice and reduces nigral activation of NF-κB, suppresses nigral expression of proinflammatory molecules and attenuates nigrostriatal degeneration in MPTP-intoxicated mice (Ghosh et al., 2007). Besides that, cholesterol lowering drug statins also protects the dopaminergic neurons in MPTP mouse model of PD and currently statin is in clinical trial II (Ghosh et al., 2009).

**Mitochondrial approached for dopaminergic neuroprotection**

Robust evidence of mitochondrial dysfunction from post-mortem brain tissue, cell culture and animal models of PD and genetic analysis of human suggests that mitochondrial dysfunction plays an important role in pathogenesis of PD. Mitochondrial dysfunction due to oxidative stress, mitochondrial DNA deletions, altered mitochondrial morphology, and interaction of pathogenic proteins with mitochondria results in dopaminergic
neurodegeneration. So, therapeutic approaches targeting mitochondrial dysfunction and related oxidative stress are of greater importance in curing PD.

*Mitochondrial dysfunction*

Various evidence suggests a role of mitochondrial dysfunction in the pathogenesis of PD (Beal, 2005; Lin and Beal, 2006). Mutations in the mitochondrial DNA (mt DNA) play a role in dopaminergic neuron demise. In addition, high levels of somatic mtDNA point mutations in elderly PD patients have also been reported (Simon et al., 2004). However, the most promising evidence of mitochondrial dysfunction in PD has been reported from the MPTP, a parkinsonian toxicant which causes parkinsonian syndromes in humans, rodents and primates by inhibiting the mitochondrial complex-I of the electron transport chain (Sandy et al., 1993). Similar to MPTP, other complex-I inhibitors such as rotenone, maneb, paraquat, fenzaquin and trichloroethylene result in loss of nigral dopaminergic neurons in mice model of PD, implicating mitochondrial dysfunction in the disease pathogenesis (Betarbet et al., 2000; Gash et al., 2008). Additionally, impairment of complex-I activity has been reported in substantia nigra, platelets and skeletal muscles of PD patients (Benecke et al., 1993; Haas, 2007). Moreover, reduced complex-I activity and an increased susceptibility to MPP⁺, the toxic metabolite of MPTP were also observed in mitochondrial DNA from PD patients, clearly demonstrating the mtDNA encoded defects in PD (Swerdlow et al., 1996; Gu et al., 1998). Recently, another convincing evidence of mitochondrial dysfunction in PD has been reported in conditional knockout ‘MitoPark’ mice. Mitopark mice have a disrupted mitochondrial tyranscription factor A (Tfam) gene in dopaminergic neurons (Ekstrand et al., 2007). These mice demonstrate reduced mtDNA expression, attenuated expression of respiratory chain function in dopaminergic neurons in nigra along with behavioral imparities
and striatal dopamine depletion, mimicking progressive PD phenotypes starting from 18 weeks of age (Ekstrand et al., 2007). Reduction of mitochondrial mass and size in the dopaminergic neurons as compared to non-dopaminergic neurons of mouse SN have been reported, suggesting important role of mitochondrial dysfunction in selective imparities in dopaminergic neurons in substantia nigra (Liang et al., 2007). Pathogenic mutations in several genes including α-synuclein, LRRK2, parkin, DJ-1 and PINK-1 also play important role in mitochondrial dysfunction in PD patients (Dauer and Przedborski, 2003; Thomas et al., 2007). Impairment of mitochondrial function in MPTP mouse model of PD has been reported in α-synuclein overexpressed cells and transgenic mice (Hsu et al., 2000; Song et al., 2004). Based on all these evidences, it could be inferred that intervening one of these processes could alleviate harmful effects of mitochondrial dysfunction. Numerous bioenergetics agent that improve mitochondrial function including creatine, CoQ10 are in clinical trial for PD. Recently Ghosh et al., (2010) have demonstrated that mitochondria targeted antioxidant Mito-Q10, which is a chemical modification of Co-Q could protect the dopaminergic neurons from MPTP-induced toxicity in mice (Ghosh et al., 2010).

**Creatine**

Creatine is a nitrogenous guanidine compound that forms high energy phosphate bonds and occurs naturally in vertebrates and supply energy to muscle and nerve cells. By a sodium-dependent creatine transporter, it is naturally taken up into the brain and skeletal muscle. It’s physiologically active form is phosphocreatine (PCr), that plays important role in maintaining energy balance in the brain. Preclinical studies in various models demonstrate its potential role as a neuroprotective agent (Gualano et al., 2010). Creatine has been shown to enhance the survival of glutamate-treated neuronal and glial cells by controlling Ras/NF-κB
Recently, neuroprotective role of creatine has been demonstrated in MPP+ and 6-OHDA treated dopaminergic neuronal culture, where it protects the tyrosine hydroxylase immunoreactive dopaminergic neuron and its fiber (Andres et al., 2005). In MPTP mouse model of PD, creatine restores MPTP-induced loss of dopamine, protects the dopaminergic neurons dose dependently (Matthews et al., 1999). Moreover, creatine in combination with nicotinamide protects against malonate-induced striatal lesions (Malcon et al., 2000). Recent studies also demonstrated that creatine and COX-2 inhibitor protects the neurons in G93A transgenic mice model of ALS (Klivenyi et al., 1999). Two grams daily creatine administration has been shown to improve behavioral difficulties in a clinical trial of 200 subjects who are within 5 years of PD diagnosis (NINDS, 2006). Although a 2-year placebo-controlled study of 60 subjects study demonstrated no improvement in patient PD scores and no effect upon dopamine transporter imaging, however, improvement of mood behavior (a nonmotor symptom of PD) was noticed in those patients (Bender et al., 2006). Recently, phase III clinical trial has been started by the NIH, where creatine is administered with a dose of 10g in a large long term study of PD targeting 1720 participants with the disease (Bloom, 2007; Couzin, 2007).

**Coenzyme Q10**

Coenzyme (Co) Q10 is an essential component for electron transport chain (ETC), where it accepts electrons from complexes I and II (Yang et al., 2009). It is also a coenzyme for complex III and serves as an important powerful antioxidant in the mitochondria that prevents oxidative damage by the free radicals (Geromel et al., 2002). CoQ10 can directly scavenge free radicals through interaction with a-tocopherol (Tiano et al., 2007). Along with its free radical scavenging activity, it also prevents apoptotic cell death by blocking Bax
binding to mitochondria and by inhibiting the mitochondrial permeability transition activation (Naderi et al., 2006). CoQ10 is also a co-factor of mitochondrial uncoupling protein (UCP) and by activating UCP, CoQ10 exerts its neuroprotective effects through a reduction in mitochondrial free radical generation (Beal, 2004). Mitochondrial UCP expression in the SN of primates is increased by CoQ10 (Schulz et al., 1995). Neuroprotective properties of CoQ10 have been demonstrated in various in vivo and in vitro model of PD. Paraquat and rotenone induced mitochondrial dysfunction and neurodegeneration in rat mesencephalic primary neurons are inhibited by CoQ10 (Moon et al., 2005). Neuroprotective role of CoQ10 has also been demonstrated in iron-induced apoptosis in dopaminergic neurons (Ono et al., 2005). Consequently, pretreatment of neuronal cells with CoQ10 maintains mitochondrial membrane potential during oxidative stress and reduces the mitochondrial generation of ROS (Somayajulu et al., 2005). In different toxin models of PD, neuroprotective role of CoQ10 has also been demonstrated. In MPTP mouse model, CoQ10 protects MPTP-induced dopamine depletion and loss of dopaminergic neurons in aged mice (Beal MF et al., 1998). Similarly, CoQ10 is also successive against MPTP toxicity in chronic MPTP model, which mimics the progressive nature of PD (Beal et al., 1998). Oral administration of CoQ10 also produces dose-dependent neuroprotective effects against malonate-induced striatal lesion and reduction of ATP (Beal et al., 1998). In the cancer chemotherapy combination of two or more drugs are really effective, similarly in neuroprotective studies combination therapy can be used. Combination of CoQ10 and minocycline in R6/2 transgenic mouse model of Huntington disease (HD) resulted in significant attenuation of neuropathological features (Stack et al., 2006). Similarly, a combination of creatine and CoQ10 exhibit significant neuroprotective effect in
MPTP mouse model of PD (Chaturvedi and Beal, 2008). A double blind, placebo controlled phase II study of CoQ10 with 3 different doses such as 300, 600 and 1200mg daily in 80 early untreated patients for 16 months was conducted recently with 1200mg dose showed some statistical improvement in PD rating scale (Shults et al., 2002). In continuation of the above mentioned phase II trial, a randomized, double blind, phase II trial of a higher dose (2400mg/daily) of CoQ10 was conducted on early untreated PD patients. Although there was some trend of improvement of clinical score by CoQ10, unfortunately, scores were not statistically significant. One possible reason could be the lower number of patients (NINDS, 2007). Recently, a NINDS-funded phase-III, multicenter, randomized, placebo controlled, double-blind trial of CoQ10 at doses of 1200 and 2400mg/daily has been initiated on 600 early, nonmedicated PD patients. Hopefully, some positive outcome will come from this clinical trial.

*Mitochondria targeted antioxidants* - Mito-Q\(_{10}\)

Mitochondria targeted antioxidants (MTAs) were shown to be more potent than nontargeted parent antioxidants in endothelial cells subjected to oxidant stress (Dhanasekaran et al., 2004). Mito-Q10 is a derivative of CoQ10 conjugated to triphenylalkylphosphonium ions, which promotes the uptake into mitochondria, where it can counteract mitochondrial ROS (Tauskela, 2007). Recent research has demonstrated that the mitochondrial uptake of antioxidants (vitamin E, coenzyme-Q) coupled to a triphenylphosphonium cation (Mito-E, Mito-Q10) is facilitated by the large negative membrane potential across the mitochondrial inner membrane (Murphy, 1997). As conventional ROS detoxification probes such as vitamin E, tempol and ubiquinone do not significantly accumulate within mitochondria. Their ability to scavenge mitochondrial reactive oxygen species (ROS) / reactive nitrogen...
species (RNS) is limited. Previous studies demonstrated the intramitochondrial concentrations of MTAs can be 100- to 500-fold higher than the cytosolic levels (Smith et al., 1999; Smith et al., 2003). The large negative membrane potential of 150–180 mV across the mitochondrial inner membrane potentiates the uptake and distribution of lipophilic cations from the intracellular space into the mitochondria (Smith et al., 2003). Similarly, substantial decrease in the levels of Co-Q, an antioxidant and critical cofactor in the mitochondrial respiratory chain, is reported animal model of PD (Lovenberg et al., 1979; Shults et al., 1999). Treating mesencephalic dopaminergic neurons with high level of chemically modified, targeted Co-Q, such as Mito-Q10, therefore, seems a logical neuroprotective strategy. We recently demonstrate the efficacy of Mito-Q10 in neuroprotection both in cell culture and in pre-clinical animal model of PD (Ghosh et al., 2010). Mito-Q10 protects the MPP+ induced loss of neurons and neurites in a cell culture model of PD. Mito-Q10 at a dose of 4 mg/ kg/ day protects the MPTP-induced loss of dopaminergic neurons and terminals in nigrostriatum and restores MPTP-induced loss of dopamine and its metabolites (Ghosh et al., 2010). Along with that, it also significantly improves MPTP-induced loss of behavioral activities (Ghosh et al., 2010). The ability of Mito-Q10 to function as an antioxidant is related to the redox reaction between the oxidized and the reduced forms of Mito-Q10 and ROS/RNS. MTAs exert their antioxidant mechanism of action via several pathways. Mito-Q10, a redox mixture of quinone and hydroquinone, undergoes reduction in the mitochondrial matrix to form the quinol form (reduced form), which is presumably the active form of the drug. The quinol form is oxidized back to quinone by ROS/RNS including lipid peroxyl radical, hydroxyl radical, superoxide, and peroxynitrite (James et al., 2005). Known mitochondria targeted Mito-quinol reacts slowly
with superoxide, Mitoquinone reacts rapidly with superoxide. Mito-Q10 mainly reacts as Mito-quinol with lipid peroxidation intermediates (James et al., 2005; Murphy and Smith, 2007).

**Table 4. Mitochondria targeted Neuroprotective agents in animal and human clinical trials**

<table>
<thead>
<tr>
<th>Mitochondria targeted compound</th>
<th>Characteristics</th>
<th>Model systems</th>
<th>Results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatine</td>
<td>Mitochondrial stabilizer; ATP synthesis enhancer</td>
<td>MPTP mouse model; Human clinical trial</td>
<td>Protected against MPTP-induced dopamine depletion; delayed progression of PD by 50%; currently in phase III trial</td>
<td>(Matthews et al., 1999; NINDS, 2006)</td>
</tr>
<tr>
<td>Coenzyme Q10</td>
<td>Antioxidant and mitochondrial enhancer</td>
<td>MPTP mouse model; Human clinical trial</td>
<td>Protected nigrostriatal dopaminergic neurons; prevented dopamine depletion in striatum; delayed progression of PD in patients with no adverse effects; neuroprotection in PD patients</td>
<td>(Shults et al., 1999; Shults et al., 2002; NINDS, 2007)</td>
</tr>
<tr>
<td>Creatine + CoQ10</td>
<td>No Human clinical trial yet</td>
<td>Combination more effective in chronic model- showing neuroprotection</td>
<td>(Yang et al., 2009)</td>
<td></td>
</tr>
<tr>
<td>Mitoquinone</td>
<td>Derivative of CoQ10; Mitochondria targeted antioxidant</td>
<td>MPTP mouse model; human clinical trial</td>
<td>Showed dopaminergic neuroprotection in SN; restored striatal dopamine; protected behavioral activities; Phase II clinical trial demonstrated it didn’t alter disease progression</td>
<td>(Ghosh et al., 2010; Snow et al., 2010)</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>Precursor of NADH, a substrate for complex-I</td>
<td>MPTP mouse model of PD; No clinical trial yet</td>
<td>Prevented neurodegeneration in mice</td>
<td>(Schulz et al., 1995)</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>Involve in mitochondrial biogenesis</td>
<td>MPTP mouse model of PD; No clinical trial yet</td>
<td>PGC-1α null mice susceptible to MPTP toxicity; Promising therapeutic target</td>
<td>(McGill and Beal, 2006; St-Pierre et al., 2006)</td>
</tr>
</tbody>
</table>

In brain, manganese superoxide dismutase (MnSOD) is a predominant anti-oxidant enzyme, but in neurological distress conditions cellular antioxidant systems including MnSOD are...
severely affected (Schwartz, 1996). The key issue in MTA-mediated antioxidant therapy is whether MTA exerts its protective action at mitochondria, the target point. Mito-Q10 at a dose of 40 - 80 mg/ kg when administered via oral gavage prevents liver damage in hepatitis C patients (Gane et al., 2010). Currently, phase II clinical trial of Mito-Q10 treatment for PD in New Zealand is initiated (Snow et al., 2010).
CHAPTER II

ACTIVATION OF NOVEL PRO-APOPTOTIC PROTEIN PIN1 (PEPTIDYL-PROLYL CIS/TRANS ISOMERASE) – RELEVANCE TO THE PATHOGENESIS OF PARKINSON’S DISEASE

A manuscript to be submitted to The Journal of Neuroscience

Anamitra Ghosh¹, Hariharan Saminathan¹, Arthi Kanthasamy¹, Vellareddy Anantharam¹, Ajay Rana², Anumantha G. Kanthasamy¹

¹Department of Biomedical Sciences, Iowa Center for Advanced Neurotoxicology, Iowa State University, Ames, IA 50011, USA; ²Department of Pharmacology, Loyola University, Chicago, IL 60141

Abstract

Parkinson’s disease (PD) is the second most common neurodegenerative disease characterized by a slow and progressive degeneration of dopaminergic neurons in substantia nigra (SN). Pathophysiological mechanisms underlying the progressive degenerative process in PD is still unclear. Pin1, a peptidyl prolyl isomerase, plays important pathophysiological roles in cancers and Alzheimer’s disease, whereas the role of Pin1 in dopaminergic neurodegeneration in PD is yet unknown. We sought to systematically characterize the role of Pin1 in PD model. Exposure of MN9D cells to MPP⁺ induced a stochastic increase in Pin1
mRNA and protein levels. Additionally, siRNA mediated knockdown of Pin1 almost completely prevented MPP⁺-induced caspase-3 activation and DNA fragmentation, confirming that Pin1 play a proapoptotic role in dopaminergic neuronal cells. Paradoxically, Juglone, the pharmacological inhibitor of Pin1, attenuated MPP⁺-induced Pin1 upregulation. Juglone also attenuated MPP⁺-induced cell death and caspase-3 activation. We also examined the changes to Pin1 expression in a MPTP-induced C57BL/6 mouse model of PD and we found MPTP injection resulted in an increased expression of Pin1 both in the SN and striatum of mice. Also, Pin1 co-localized with tyrosine hydroxylase in the dopaminergic neurons in SN of human PD patients and MPTP treated mice. Furthermore, Juglone inhibited MPTP-induced Pin1 expression in the nigrostriatum. Additionally, Juglone improved motor function, normalized striatal neurotransmitters and protected dopaminergic neurons in MPTP treated mice, suggesting that Juglone is capable of neuroprotection in the MPTP mouse model. Collectively, our findings demonstrate for the first time that Pin1 is upregulated and has neurodegenerative roles in cell culture and animal models of PD. Also, Pin1 inhibitor Juglone could be an effective neuroprotective agent in management of PD.

Introduction

Parkinson’s disease (PD) is the most common neurodegenerative movement disorder affecting 1% of population over 65 years of age (Lees et al., 2009). It is chronic and progressive in nature and characterized predominantly by resting tremors, muscular rigidity, bradykinesia and postural instability along with several non-motor symptoms (Savitt et al., 2006). Pathologically it is characterized by gliosis, progressive loss of dopaminergic neurons and their terminals in nigrostriatal axis, and appearance of cytoplasmic inclusions known as
Lewy bodies in surviving neurons of substantia nigra (SN) (Dunnett and Bjorklund, 1999; Dauer and Przedborski, 2003; Ghosh et al., 2009).

Reversible phosphorylation on Ser/Thr – Pro (S/T-P) motifs regulated by Pro-directed protein kinases and phosphatases is an important molecular switch in controlling various cellular processes (Gothel and Marahiel, 1999; Lu, 2000; Jager et al., 2006). Proline directed kinases such as cyclin-dependent protein kinases, extracellular signal-regulated kinases, stress-activated protein kinases/c-Jun-N-terminal kinases, p38 MAP kinases, glycogen synthase kinase – 3, Polo-like kinases and some of the Ser/Thr phosphatases phosphorylate or dephosphorylate such motifs to regulate cellular signaling (Sudol et al., 2001; Shaw, 2007). Since the discovery of protein interacting with never in mitosis – A (Pin1), a lot has been known in the conformational importance of the Pro-directed kinases in cellular signaling (Uchida et al., 1999). Pin1 is unique among the peptidyl-prolyl isomerases, in that, they specifically recognize phosphorylated serine or threonine residues immediately preceding proline (pSer/Thr-Pro) in a subset of proteins and isomerizes the cis/trans conformation of the peptide bond (Joseph et al., 2003; Lu, 2004). Numerous studies have shown that Pin1 catalyzed conformational regulation have a profound impact in the regulation of cell growth, stress responses, immune responses, germ cell development, neuronal differentiation and survival (Atchison and Means, 2004; Braithwaite et al., 2006). Deregulation of Pin1 signaling is implicated in a growing number of pathological conditions such as Alzheimer’s disease (AD) (Akiyama et al., 2005; Maruszak et al., 2009), asthma (Esnault et al., 2008; Shen et al., 2008), frontotemporal dementia (Thorpe et al., 2004), Parkinsonism linked to chromosome 17 (FTDP-17) (Yotsumoto et al., 2009), Pick disease (Ramakrishnan et al., 2003), progressive supranuclear palsy (PSP), corticobasal degeneration
(CBD) (Hutton et al., 2001; Lee et al., 2001) and cancer (Li et al., 2006). High expression levels of Pin1 in terminally differentiated and post mitotic neurons suggest that it plays important roles in neurons except cell cycle regulation and proliferation (Lu et al., 1999; Becker and Bonni, 2006). In neurodegeneration, Pin1 interacts with mitochondrial – BH3-only protein BIMEL and activates c-JUN to regulate a neuron-specific activation of mitochondrial apoptotic machinery (Becker and Bonni, 2007). Additionally, mitochondrial respiratory chain function, oxidative stress and calcium handling capacities cause selective degeneration of dopaminergic neurons in PD (Kanthasamy et al.; Vila et al., 2001). Recently Ryo et al., reported that Pin1 overexpression facilitates formation of α-synuclein inclusions in a cellular model of α-synuclein aggregation and Pin1 also localizes in Lewy bodies in PD patients (Ryo et al., 2006). But Pin1’s level, activity and function in the pathogenesis of PD are completely unknown.

Herein, we report for the first time to our knowledge that, there is a dopaminergic neuron specific upregulation of Pin1 in a MPTP model of PD. We have shown that expression of Pin1 increases in MPP+ -treated cell culture model and MPTP treated mice. Also, Pin1 functions as an important pro-apoptotic factor in selective degeneration of dopaminergic neurons. Furthermore, Pin1 inhibitor Juglone attenuates MPTP induced Pin1 expression in vitro and in vivo. We also demonstrate that Juglone protects against MPTP induced neurodegeneration in nigrostriatal axis. Collectively, our results indicate that Pin1 could be a possible therapeutic target in the management of PD.

Materials and Methods

Chemicals and biological reagents. 1-Methyl-4-phenyltetrahydropteridine (MPP+ iodide) and MPTP-HCl were purchased from Sigma (St. Louis, MO, USA). Pin1 inhibitor Juglone
was purchased from Calbiochem. Caspase substrate (Ac-DEVD-AFC) was obtained from Bachem Biosciences (King of Prussia, PA). Bradford protein assay reagent was purchased from Bio-Rad Laboratories (Hercules, CA). Neurobasal medium, B27 supplement, fetal bovine serum, L-glutamine, penicillin and streptomycin was purchased from Invitrogen (Carlsbad, CA).

**Cell culture.** MN9D dopaminergic cell line originated upon fusion of rostral mesencephalic neurons from embryonic C57BL/BJ (embryonic day 14 mice) with N18TG2 neuroblastoma cells (Choi et al., 1991). MN9D cells were grown in a high-glucose (4500 mg/L) Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma-Aldrich, St. Louis, MO) containing 10% Tet-approved fetal bovine serum (Invitrogen, Carlsbad, CA), 3.7 g L\(^{-1}\) NaHCO\(_3\) and 4mM l-glutamine in a 5% CO\(_2\) atmosphere at 37°C.

**Treatment paradigm.** MPP\(^+\) (300μM) was added to the cells for the duration of the experiment. The cells were detached from the flask using a rubber policeman and centrifuged at 200 x g for 5 min, washed with ice-cold PBS twice and homogenized with Radio-immunoprecipitation Assay (RIPA) buffer. Cell lysates, collected by spinning down the cell fragments at 20,000 x g for 45 min at 4°C, were used to determine changes to Pin1 expression.

**siRNA transfection.** MN9D cells were plated in 6-well tissue culture plates at 70-75% confluency. Transfections were performed using Lipofectamine\(^\text{TM}\) 2000 (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. Four to six hours after transfection, the media was replaced with fresh growth media. The following day, cells were treated with MPP\(^+\) or vehicle and processed for further experiments.
**qRT-PCR.** After treatment, total RNA were extracted from the MN9D cells or the mice brain SN using Absolutely RNA Miniprep Kit (Agilent Technologies, Santa Clara, CA) following the manufacturer’s protocol. Total RNA was treated with DNase I to remove DNA contamination and then reversibly transcribed into first-strand cDNA using the SuperScript III first-strand synthesis system (Invitrogen, Carlsbad, CA) as described in the kit instructions. SYBR-green Quantitative PCR was performed with validated primers of Pin1 and control β-actin primers (Qiagen, Valencia, CA) using either FastStart Universal SYBR green master (Rox) (Roche Applied Science, Indianapolis, IN) for the MN9D cells or RT² SYBR® Green qPCR Master Mix (SABiosciences, Fredrick, MD) for the mice tissue.

**Sytox cell death assay.** The MN9D cells were incubated with 300µM MPP⁺ for upto 24 h in the presence or absence of 1µM Juglone and cell death was determined by the cell impermeable Sytox green (Molecular Probes, Eugene, OR). Sytox green intercalates with the DNA in the membrane compromised cells to produce a green fluorescence that is quantifiable at excitation 485 nm and emission 538 nm using the fluorescence microplate system (Synergy 4, Biotek, Winooski, VT) and also allows to be viewed under fluorescence microscope. Fluorescence and phase contrast were taken after exposure to MPP⁺ with a NIKON TE2000 microscope, and pictures were captured with a SPOT digital camera.

**Caspase-3 activity assay.** Caspase-3 activity was determined as previously described (Kitazawa et al., 2004; Anantharam et al., 2007). After the MPP⁺ treatment, cells were lysed, centrifuged and the resulting supernatants were incubated with 50µM Ac-DEVD-AMC (a caspase-3 substrate) at 37°C for 1 h and caspase activity was measured by fluorescence plate reader (Synergy 4, Biotek, Winooski, VT) with excitation at 380 nm and emission at 460 nm.
**DNA fragmentation assay.** DNA fragmentation was measured using a Cell Death Detection ELISA Plus Assay Kit as described previously (Song et al., 2010; Afeseh Ngwa et al., 2009). After MPP+ treatment, cells were centrifuged and washed with PBS once. DNA fragmentation was measured in the cell lysates according to the manufacturer’s protocol. The absorbance of the ELISA reaction was measured at 490 and 405 nm using a microplate reader (SpectraMAX 190, Molecular Devices Corp., Sunnyvale, CA).

**High-Affinity [3H] Dopamine Uptake Assay.** Cells in each well were washed twice with 1 ml of Krebs-Ringer buffer (16 mM NaH₂PO₄, 16 mM Na₂HPO₄, 119 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl₂, 1.2 mM MgSO₄, 1.3 mM EDTA, and 5.6 mM glucose; pH 7.4). The cells were then incubated with 25 nM [3H]dopamine in Krebs-Ringer buffer (0.4 ml/well) for 20 min at 37°C. Nonspecific uptake of dopaminergic was determined in parallel wells incubated with tritiated dopamine and 10 μM mazindol, an inhibitor of neuronal dopamine uptake. Briefly, the cells were washed three times with ice-cold Krebs-Ringer buffer (1 ml/well) and lysed with 1 N NaOH (0.5 ml/well). After washing the lysates were mixed with 5 ml of scintillation fluid (Scintiverse BD), radioactivity was determined with a liquid scintillation counter (Tri-Carb 1600 TR; Packard, Meriden, CT). The specific dopaminergic uptake was calculated by subtracting the amount of radioactivity observed in the presence of mazindol from that observed in the absence of mazindol.

**Animals and MPTP treatment.** Eight- to 12-week-old male C57BL/6 mice weighing 24 to 28 g were housed in standard conditions: constant temperature (22 ± 1°C), humidity (relative, 30%), and a 12-h light/dark cycle. Mice were allowed free access to food and water. Use of the animals and protocol procedures were approved and supervised by the Committee on
Animal Care at Iowa State University (Ames, IA, USA). Mice received either a single high dose of MPTP-HCl (30mg/kg free base) or four injections of MPTP-HCl (15mg/kg of free base; Sigma-Aldrich) in 1XPBS at 2 h intervals via i.p. The control mice received only 1XPBS.

**Juglone treatment in mice.** Juglone was dissolved in 10% ethanol and was administered to mice via intraperitoneal injection. Based on other reports, (Esnault et al., 2007; Jeong et al., 2009) we injected 10mg/kg dose of Juglone intraperitoneally. However, we observed nearly 40% mortality rate of mice. Hence, we chose a lower dose (3mg/kg) of Juglone and we never noticed any death or other behavioral impairities in mice. The first dose of Juglone (3mg/kg) was administered 24 h before 1st dose of MPTP injection, the second dose of Juglone was administered 3 h before the 1st dose of MPTP injection and the third dose of Juglone was administered 3 h after the last dose of MPTP injection. For neuroprotection studies, mice were treated for additional 6 days of Juglone treatment with a single dose each day.

**Immunoblotting.** Cells were collected after exposure to MPP⁺, resuspended in RIPA buffer and brain tissues after MPTP treatment were resuspended in modified RIPA buffer, both containing protease and phosphatases inhibitor cocktail. Cell suspensions were sonicated after resuspension, while tissues were homogenized, sonicated and then centrifuged at 14000 x g for 1 hr at 4°C. Lysates were separated on 8-12% SDS – polyacrylamide gel electrophoresis (PAGE). After the separation, proteins were transferred to a nitrocellulose membrane, and nonspecific binding sites were blocked by treating with Odyssey® blocking buffer (LiCor, Lincoln, NE). Pin1 (1:2000; mouse monoclonal; R&D Systems, Minneapolis, MN), TH (1:1500, mouse monoclonal; Millipore, Billerica, MA), β-Actin (1:5000; mouse
monoclonal; Sigma, St. Louis, MO) were used to blot the membranes. Secondary IR-680-conjugated anti-mouse (1:10000, donkey anti-mouse; Molecular Probes, Carlsbad, CA) was used for antibody detection with the Odyssey IR imaging system (LiCor, Lincoln, NE).

**Mesencephalic Primary Neuron Cultures and Treatment.** Primary mesencephalic and striatal neuronal cultures were prepared from the ventral mesencephalon of gestational 14- to 15-day-old mice embryos as described previously (Zhang et al., 2007). Briefly, mesencephalic and striatal tissues from E14 to 15 mouse embryos were dissected and maintained in ice-cold calcium-free Hanks’ balanced salt solution and then dissociated in Hanks’ balanced salt solution containing trypsin-0.25% EDTA for 20 min at 37°C. The dissociated cells were then plated at equal density of 0.6 million cells per well on 12-mm coverslips precoated with 0.1 mg/ml poly-D-lysine. Cultures were maintained in neurobasal medium fortified with B-27 supplement, 500 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA). The cells were maintained in a humidified CO₂ incubator (5% CO₂ and 37°C) for 24 h. Half of the culture medium was replaced every 2 days. Approximately 6- to 7-day-old cultures were used for experiments. Primary mesencephalic and striatal dopaminergic neuronal cells were exposed to 10 μM MPP⁺ in the presence or absence of Juglone (1 μM) for 24 h.

**Immunocytochemistry.** The primary mesencephalic and striatal neurons were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 20 minutes and processed for immunocytochemical staining. First, nonspecific sites were blocked with 2% bovine serum albumin, 0.5 % Triton X-100 and 0.05% Tween-20 in PBS for 45 min at room temperature. Cells were then incubated with different primary antibodies such as TH (1:1600, mouse
monoclonal; Millipore, Billerica, MA), Pin1 (1:400, goat polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. Appropriate secondary antibodies (Alexa Fluor 488 or 594 or 555; Invitrogen) were used followed by incubation with 10µg/ml Hoechst 33342 (Invitrogen) for 5 min at room temperature to stain the nucleus. The coverslip containing stained cells were washed twice with PBS and mounted on poly-D lysine coated slides (Sigma, St Louis). Cells were viewed under a NIKON inverted fluorescence microscope (model TE-2000U; NIKON, Tokyo, Japan) and images were captured with a SPOT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

**Quantification of TH positive cell counts and neuronal processes.** Tyrosine hydroxylase positive dopaminergic neurons and neuronal processes in primary neuronal cultures from each coverslip were measured using MetaMorph software, version 5.0 (Molecular Devices, Sunnyvale, CA) as described previously (Zhang et al., 2007). For measurement of TH cell count, pictures were first taken at 20X lens, then thresholded. After that, neurons were counted using the Integrated Morphometry Analysis function. For measurement of neuronal processes, pictures were taken using 60X oil immersion lens and the lengths of the processes were marked by applying the region and length measurement function in the Integrated Morphometry Analysis. TH positive neurons and their processes were counted in at least six individual cultures for each treatment.

**Diaminobenzidine immunostaining and stereological counting of TH positive neurons.** Tyrosine hydroxylase and Pin1 diaminobenzidine (DAB) immunostaining were performed in striatal and substantia nigral sections as described previously (Ghosh et al., 2009). Briefly, seven days after MPTP injection, mice were sacrificed and perfused with 4%
paraformaldehyde (PFA) in 1X PBS and post-fixed with PFA and 30% sucrose. The fixed brains were subsequently cut using a cryostat into 30 µm coronal sections in 30% sucrose-ethylene glycol solutions at -20°C. On the day of staining, sections were first washed with PBS and incubated with the anti-TH antibody (1:1600, rabbit anti-mouse; Calbiochem, Gibbstown, NJ) or with the anti-Pin1 antibody (1:400, goat anti-mouse; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. Biotinylated anti-rabbit or anti-goat secondary antibodies were used for 2 h at room temperature. The sections were then incubated with avidin peroxidase (Vectastatin ABC Elite kit, Burlingame, CA) for 45 min at room temperature. Immunolabeling was observed using DAB which yielded a brown colored stain. Total numbers of TH-stained neurons in SN were counted stereologically with Stereo Investigator software (MicroBrightField, Inc., Williston, VT, USA), using an optical fractionators (Ghosh et al., 2009).

**HPLC analysis of striatal dopamine and its metabolites levels.** The striatal dopamine (DA), DOPAC and HVA levels were quantified using a high-performance liquid chromatography (HPLC) with electrochemical detection. Samples were prepared and quantified as described previously (Ghosh et al.). Briefly, 7 days after MPTP treatment, mice were sacrificed and striata were collected and neurotransmitters from striatal tissues were extracted in 0.1 M perchloric acid solutions containing 0.05% Na₂EDTA and 0.1% Na₂S₂O₅) and isoproterenol (as an internal standard). The extracts were filtered in 0.22 µm spin tubes, and 20 µl of the sample was loaded for analysis. Dopamine and its metabolites (DOPAC and HVA) were separated isocratically in a C-18 reversed-phase column using a flow rate of 0.6 ml/min. A HPLC system (ESA Inc., Bedford, MA, USA) with an automatic sampler
equipped with a refrigerated temperature control (model 542; ESA Inc.) was used in these experiments. The electrochemical detection system consisted of a Coulochem-I model 5100A with an analytical cell (model 5014A) and a guard cell (model 5020) (ESA Inc.). The data acquisition and analysis were performed using the EZStart HPLC Software (ESA Inc.).

**Immunohistochemistry.** Twenty four hours after MPTP treatment, mice were perfused with 4% paraformaldehyde and post fixed with PFA and 30% sucrose respectively. Next, 30 µm coronal SN free-floating sections were blocked with 2% bovine serum albumin, 0.5 % Triton X-100 and 0.05% Tween-20 in PBS for 1 h at room temperature. Sections were incubated with different primary antibodies such as anti- Pin1 antibody (1:500; goat polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA), anti-NeuN (1:500; mouse monoclonal; Chemicon, Temecula, CA), anti-TH (1:1600; mouse monoclonal; Chemicon, Temecula, CA) for overnight at 4°C. After washing with PBS, sections were incubated in appropriate secondary antibodies (Alexa Fluor 488 or 594 or 555 from Invitrogen) for 2 h. followed by incubation with 10µg/ml Hoechst 33342 for 5 min at room temperature to stain the nucleus. Sections were viewed under a NIKON inverted fluorescence microscope (model TE-2000U; NIKON, Tokyo, Japan); images were captured with a SPOT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) using MetaMorph software, version 5.0 (Molecular Devices, Sunnyvale, CA).

**Behavioral measurements.** We performed open-field experiment for testing the locomotor activities (Ghosh et al.; Zhang et al., 2007) and the rotarod experiment (Ghosh et al.; Gianni et al., 2009) to test feet movement of mice after MPTP and Juglone treatments. An automated device (AccuScan, model RXYZCM-16, Columbus, OH, USA) was used to measure the
spontaneous activity of mice. The activity chamber was 40×40×30.5 cm, made of clear Plexiglas and covered with a Plexiglas lid with holes for ventilation. The Infrared monitoring sensors were located every 2.54 cm along the perimeter (16 infrared beams along each side) and 2.5 cm above the floor. Two additional sets of 16 sensors were located 8.0 cm above the floor on opposite sides. Data were collected and analyzed by a VersaMax Analyzer (AccuScan, model CDA-8, Columbus, OH, USA). Before any treatment, mice were placed inside the infrared monitor for 10 min daily for 3 consecutive days to train them. Five days after the last MPTP injection, both open-field and rotarod experiments were conducted. In open-field experiment, mice were monitored for horizontal activity, vertical activity, total distance travelled (cm), total movement time (s), total rest time (s) and rearing activity over a 10 min test session. Using Versaplot and Versadat software we analyzed the data among the three groups. In rotarod, feet movement of mice was observed using a constant 20 rpm speed. Mice were given a 5-7 min rest interval to eliminate stress and fatigue.

**Statistical analysis.** Data were analyzed with Prism 3.0 software (GraphPad Software, San Diego, CA). Bonferroni and Dunett multiple comparison testing were used. Differences with P<0.05 were considered significant.

**Results**

**MPP⁺ induces activation of Pin1 in MN9D dopaminergic neuronal cells**

Accumulation of Pin1 in the Lewy bodies of PD and promotion of α-synuclein aggregates during over-expression of Pin1 in a kidney cell line has left a lot of unanswered questions about the role of Pin1 during PD (Ryo et al, 2006). To specifically address the role
of Pin1 in a PD model, we utilized highly relevant *in vitro* model - MN9D mouse dopaminergic neuronal cells and *in vivo* model - C57 black mice for our studies. Initially, we sought to understand changes to Pin1 message and protein levels upon treatment with Parkinsonian toxicant MPP+. We treated MN9D dopaminergic cells with 300μM MPP+ and assayed the cells at different time points. To examine the changes to Pin1 message levels, we first performed qRT PCR. As evident from Figure 1 A and B, MPP+ induced Pin1 message expression in MN9D dopaminergic cells stochastically with a maximal expression at 3 h \((p<0.001)\). Next, we examined whether Pin1 mRNA induction translated to changes in Pin1 protein levels during MPP+ treatment. We examined changes to Pin1 protein levels by immunoblotting. Figure 2 A and B, demonstrates that Parkinsonian toxicant MPP+ induced Pin1 protein expression detectable by immunoblotting in the lysates of MN9D mouse dopaminergic cells starting from 6 h with statistically significant differences at 12 h \((p<0.001)\). Together, these results demonstrate that Parkinsonian toxicant MPP+ induces Pin1 expression in MN9D dopaminergic cells.

**Juglone rescues MN9D dopaminergic cells from MPP+ induced apoptosis**

Neuroinflammation and apoptosis herald the hallmarks of PD pathogenesis. Mitochondrial impairment, activation of the mitochondrial apoptotic machinery and the caspases are well established events that culminate in the selective degeneration of dopaminergic neurons. Accumulation of Pin1 in the Lewy bodies of degenerating dopaminergic neurons of PD patients and the reports of Pin1 in the activation of neural-specific mitochondrial apoptotic machinery necessitates a thorough investigation of the function of this protein during PD. To assess whether Pin1 has a pro-apoptotic or an anti-
apoptotic function, we used the pharmacological inhibitor of Pin1 – Juglone during the treatment of MN9D mouse dopaminergic cells with Parkinsonian toxicant MPP⁺ for monitoring changes to apoptotic events. To examine the changes to Pin1 expression in the presence of Juglone, we co-treated MN9D dopaminergic cells with MPP⁺ for different time points and separated cell lysates on SDS-PAGE and immunoblotted for Pin1. To our surprise, we found that MPP⁺ induced Pin1 expression was significantly attenuated in the presence of Juglone (Fig. 2 A). We measured cytotoxicity of MN9D cells using a live-cell impermeable fluorescent dye – Sytox green during treatment of MPP⁺ in the presence or absence of Pin1 inhibitor – Juglone. Pin1 inhibition protected MN9D dopaminergic cells from MPP⁺- induced cytotoxicity (Fig. 2 B and C). Further, we wanted to examine changes to MPP⁺ induced caspase-3 activation during Pin1 inhibition. Juglone significantly attenuated MPP⁺ induced caspase-3 activation in the MN9D dopaminergic cells (Figure 2 D). Together, these results suggest that Pin1 could be a pro-apoptotic factor in the MN9D dopaminergic neuronal cells during MPP⁺ induced apoptosis. Pin1 inhibitor Juglone is also an efficient inhibitor of its expression.

Silencing of Pin1 expression ameliorates MPP⁺ induced apoptotic events

Because Pin1 inhibitor Juglone attenuated apoptotic events in an in vitro model of PD, we sought to examine whether Pin1’s pro-apoptotic function is specific by employing genetic tools. We knocked down Pin1 expression with a Pin1-specific siRNA and examined changes to apoptotic markers – caspase-3 activation and DNA fragmentation. MN9D dopaminergic cells were transfected with a Pin1 specific siRNA. Knock-down of Pin1 expression could be examined only after treatment of MPP⁺. As shown in Figure 3A, we
could knock-down MPP⁺ induced Pin1 expression effectively in the Pin1 specific siRNA transfected cells. Knock-down of Pin1 by specific siRNA effectively attenuated apoptotic events – caspase-3 activation (Figure 3 B) and the terminal event of apoptosis – DNA fragmentation (Figure 3 C). Taken together, by employing pharmacological and genetic tools to inhibit Pin1, we show for the first time to our knowledge that Pin1 has a pro-apoptotic role in PD pathogenesis.

**Juglone inhibits MPP⁺ - induced expression of Pin1 in primary mesencephalic cultures**

Next, we checked for the expression of Pin1 in primary mesencephalic neuronal culture and role of Juglone in attenuating the Pin1 expression. Primary mesencephalic and striatal cultures were isolated from E15 mouse embryos and were cultured and grown on laminin-coated coverslips. The neuronal cultures were treated with 10μM MPP⁺ in the presence or absence of 3μM Juglone for 24 h and immunocytochemistry was performed for Pin1 and tyrosine hydroxylase (TH). As evident from Fig. 4 A and B, MPP⁺ induced increased expression of Pin1 both in primary mesencephalic and striatal cultures. However, TH+ve dopaminergic neurons in MPP⁺ treatment also expressed Pin1 in cytosol (Fig. 4A). In addition to that, we also found TH+ve dopaminergic neurons are expressing Pin1 in primary striatal cultures (Fig. 4B). Evidences of presence of dopaminergic neurons in striatum have been reported by many investigators in different species (Mura et al., 1995; Jollivet et al., 2004; Huot et al., 2007). Similar to Presna et al. (2000), we also detected majority of striatal TH+ ve neurons are expressing glutamic acid decarboxylase (GAD) (supplementary figure 1). Additionally, GAD producing neurons which are also producing dopamine are expressing Pin1 in MPP⁺ treatment (supplementary Fig.1). However, we could not detect expression of
Pin1 in dopaminergic neurons in MPP⁺ and Juglone co-treatment both in primary mesencephalic nigral and striatal culture (Fig. 4 A and B). After confirming the role of Juglone in attenuating Pin1 expression, we were interested to check if Juglone rescued dopaminergic neurons from MPP⁺ induced neurodegeneration. We measured the lengths of processes of TH +ve neurons from mesencephalic (Fig. 4 C) and striatal (Fig. 4 D) cultures and found that MPP⁺ and Juglone co-treated dopaminergic neuronal processes are significantly longer than the processes of neurons treated only with MPP⁺ (Fig. 4 C and 4 D). Integrity of dopaminergic neurons is always correlated positively with dopamine reuptake capacity and serves as a functional indicator of the healthiness of the neurites (Liu et al., 2000). Primary mesencephalic cultures were exposed to 5µM MPP⁺ in the presence or absence of 3µM Juglone for 48 h. MPP⁺-Juglone co-treated cells were significantly protected from MPP⁺-induced loss of [³H] dopaminergic reuptake (Fig. 4 E). Taken together, these results clearly demonstrate that Juglone has a neuroprotective effect during MPP⁺ toxicity and also suggest that the dopamine transporters may be intact during Juglone co-treatment.

MPTP activates Pin1 expression and activity in the Substantia nigra of mice

Our earlier findings demonstrate the selective upregulation of Pin1 in the in vitro models of PD (Fig. 1). Next, we sought to examine whether MPTP induces Pin1 activation in the mice brain. First, we checked for the expression of Pin1 at different time points after a single high dose of MPTP (30mg/kg). We observed the highest expression of Pin1 in SN (Fig 5 A and B) and the STR (Fig. 5 C and D) 6 h after MPTP injection. The magnitude of Pin1 expression in nigrostriatum decreased 12h after MPTP injection. There was little expression
of Pin1 in nigrostriatum of saline treated control mice. To further confirm that MPTP induced Pin1 expression was paralleled with changes to Pin1 activity, we performed Pin1 assay in mice SN and STR. As expected, we noticed MPTP-induced a significant increase of Pin1 activity in SN in consonance with the time point where we observed a maximal expression of Pin1 (Fig. 5 E). However, there was no significant difference in Pin1 activity in the STR between control and MPTP treated mice.

Juglone inhibits the expression of Pin1 in vivo in the nigrostriatum of MPTP-intoxicated mice

After observing increased expression of Pin1 with a single high dose of MPTP, we were interested to check Pin1 expression level in acute MPTP model. Mice were injected with 4 doses of MPTP on a single day spread at 2h intervals and were sacrificed at different time points. As evident from Figure 6 A, Western blotting for Pin1 showed maximum expression of Pin1 24 h after MPTP injections. The magnitude of Pin1 expression in nigrostriatum was decreased 72 h after MPTP injections. Also, there was some elevation of Pin1 expression at 12 h after MPTP injections. Densitometry analysis for band intensities also reflected that maximum expression of Pin1 occurred 24h after MPTP injections (Fig. 6 B). When we checked the expression of Pin1 in striatum at different time points after MPTP injections, we found increased expression of Pin1 starting from 6h until 72h following MPTP injections (Fig. 6 C). Densitometry analysis of bands also confirmed similar pattern of Pin1 expression (Fig. 6 D). For our further experiments, we chose 24h time point as we observed maximal expression of Pin1 at this time point. To examine the changes to Pin1 message levels, we performed qRT-PCR in MPTP and MPTP-Juglone co-treated mice (Fig. 6I). Mice were administered Juglone (3 mg / kg body wt.) intraperitoneally 24h prior to MPTP
administration. As shown by quantitative real time PCR, MPTP administration led to a marked increase in Pin1 message expression in mice SN, whereas, Juglone significantly attenuated MPTP induced Pin1 message level (Fig. 6 I). Next, we were interested to check whether Pin1 inhibitor Juglone could suppress MPTP induced Pin1 expression in nigrostriatum. Twenty four h after MPTP administration the nigrostriatum were observed for changes to Pin1 expression by Western blot. As evident from Figure 6 E-H, we observed a robust increase in Pin1 expression in both nigra and striatum. However, in MPTP-Juglone co-treated mice we found attenuation of Pin1 expression (Fig. 6 E-H). Taken together, these results clearly suggest that Juglone inhibits MPTP induced Pin1 expression in vivo. To further confirm that MPTP induces Pin1 expression and Juglone attenuates MPTP induced Pin1 expression, we performed immunohistochemistry for Pin1 in the mice SN. DAB-immunostained pictures clearly showed increased expression of Pin1 in substantia nigra pars compacta region of ventral midbrain (Fig. 7 A). Interestingly, MPTP and Juglone co-treated mice exhibited inhibition of Pin1 protein expression in SN (Fig. 7 A). Next, we speculated on the cell type that shows MPTP induced increase in Pin1 expression in SN. To address this question we did double labeling immunohistochemistry for Pin1 and NeuN (common neuronal marker) in the mice SN. We observed that some NeuN positive neurons, but, not all, co-expressed Pin1 in the cytosol (yellow color) upon MPTP treatment (Fig. 7 B), whereas, there was little or no co-localization of Pin1 and NeuN in saline treated control sections. Besides dopaminergic neurons, other neuronal subtypes and glial cells are also present in SN. To further confirm the neuronal specificity of Pin1 upregulation, we did double labeling immunohistochemistry of TH (marker for dopaminergic neuron) and Pin1 in SN. It is clearly evident from Figure 7 C, that 70-80% of TH positive dopaminergic neurons
showed significant expression of Pin1 in cytosol of MPTP treated mice. Enlarged inset pictures clearly show colocalization of TH and Pin1 in cytosol (Fig. 7 C). However, MPTP treated mice that also received Juglone show very few colocalized neurons of TH and Pin1 in SN (Fig. 7 C), which further demonstrates Juglone’s role in attenuating Pin1 expression in mice midbrain.

**Pin1 accumulates in dopaminergic neurons of human Parkinson’s disease midbrain tissue**

As we found increased expression of Pin1 in TH positive neurons of MPTP treated mice midbrain tissues, we were interested to see whether Pin1 is also upregulated in dopaminergic neurons of PD brain. We hypothesized that Pin1 may play an important role in the pathogenesis of PD. Recent studies also reported increased expression of Pin1 in human PD brains (Ryo et al., 2006). We did double labeling of TH and Pin1 in PD brain tissues and age matched control brain tissues. We observed increased expression of Pin1 in SN of PD brain and it also co-localized in the cytoplasm of TH positive dopaminergic neurons (Fig. 8 A). However, in SN of control human sections we detected healthy TH positive neurons with little or no Pin1 expression (Fig. 8 A and B). Human PD brain contains both pigmented (with neuromelanin) and non-pigmented (without neuromelanin) dopaminergic neurons. Previous reports suggested massive loss of dopamine producing pigmented neurons occurred only in SN of PD brain (Hirsch et al., 1988; Kastner et al., 1992). In our study, we noticed increased expression of Pin1 in the cytosol of pigmented dopaminergic neurons in PD brain (Fig. 8 C), whereas in non-pigmented cells less expression of Pin1 was observed (Fig. 8 D). To further confirm our findings, we checked Pin1 expression levels in SN of human PD brains by western blot. We detected robust increase of Pin1 expression in SN of PD patients than that
of the age matched control brains (Fig 8 E). Collectively, these results clearly demonstrate that Pin1 has some pathophysiological roles in the neurodegeneration of dopaminergic neurons in PD.

**Upregulation of Pin1 is specific to the nigrostriatum region**

In our next experiment, we addressed whether the upregulation of Pin1 which plays role in pathogenesis of Parkinson’s disease is specific to the nigrostriatum regions of brain. Mice received 4 i.p. injections of MPTP at 2h interval in a single day. Twenty-four hours after the last injection mice were sacrificed and cortex and hippocampus regions were dissected out. We checked the expression of Pin1 in cortex and hippocampus and found no change in expression levels of Pin1 with MPTP treatment when compared with saline treated control mice (Fig. 9 A, B). These experiments clearly suggest that Pin1 upregulation following MPTP treatment is specific to the nigrostriatum region of brain and therefore, relevant to the pathogenesis of PD.

**Juglone improves locomotor functions in MPTP-treated mice**

After elucidating Juglone’s role in attenuating Pin1 expression in MPTP treated mice we were interested to check whether Juglone plays any role in attenuating behavioral deficits caused by MPTP. Five days after MPTP injections mice were tested for locomotor activities which include openfield and rotarod experiments. Representative maps using Versaplot software (Accuscan, OH) showed mice movement in openfield experiment for 10min (Fig. 10 A). As reported previously (Ghosh et al., 2009) we also observed marked decrease of horizontal activities (Fig. 10 B), vertical activities (Fig. 10 C), total distance travelled (Fig. 10 D), total movement time (Fig. 10 E) and rearing activities (Fig. 10 F) in MPTP treated
mice. However, MPTP treated mice that also received Juglone showed significant restoration of those behavioral parameters to the levels observed in control mice (Fig. 10 A-F). In rotarod experiment also we observed similar trend. At 20 rpm rotarod speed, MPTP treated mice showed (75%) decrease in time spent on rotarod compared to control mice (Fig. 10 G). Interestingly, Juglone treatment significantly restored (50%) rotarod activities in MPTP treated mice (Fig. 10 G). Collectively, these results demonstrate that Juglone restored the motor function impairments in MPTP treated mice.

**Juglone increases striatal dopamine and its metabolites levels in MPTP treated mice**

After establishing the role of Juglone in improving motor impairments, we next determined whether Juglone protects neurotransmitter levels during MPTP-toxicity. Mice were treated with Juglone (3 mg/kg body weight) via i.p. one day before MPTP injection and continued for another 8 days. Seven days after MPTP injections mice were sacrificed and STR tissue samples were processed for neurotransmitter analysis by HPLC (as mentioned in materials and methods). We observed ≈ 84% decrease in dopamine levels (Fig. 11 A), ≈ 90% decrease in dihydrophenylacetic acid (DOPAC) levels (Fig. 11 B) and ≈ 90% attenuation in homovanillic acid (HVA) levels (Fig. 11 C) in MPTP treated mice. In contrast, treatment of MPTP-injected mice with Juglone increased dopamine levels by ≈ 30% in comparison to MPTP alone (Fig. 11 A; p < 0.01). Additionally, Juglone increased DOPAC and HVA levels by ≈ 33% (Fig. 11 B; p < 0.01) and ≈ 18% (Fig. 11 C; not significant), respectively. These results suggest that Pin1 could be pharmacological target for the management of PD.

**Juglone protects nigrostriatal dopaminergic neurons from MPTP-induced neurodegeneration in vivo**
As Juglone significantly protected from striatal neurotransmitter loss in MPTP treated mice, we next wanted to determine whether Juglone could protect nigrostriatal axis against MPTP toxicity. Mice were treated with Juglone (3 mg/kg body weight) 24 h prior to MPTP injections and continued for another 8 days following MPTP injections. Seven days after MPTP treatment mice were sacrificed and tyrosine hydroxylase (TH) DAB immunostaining were performed in SN regions of ventral midbrain (Fig. 12). Similar to a recent article (Ghosh et al., 2009), we also observed loss of TH positive neurons in STR (Fig. 12 A) and SN (Fig. 12 B) regions of MPTP treated mice. Higher magnified (10X) pictures clearly showed MPTP induced loss of TH positive dopaminergic neurons mainly in substantia nigra lateralis (SNI), substantia nigra pars compacta (SNc) and substantia nigra ventricularis (SNr) regions (Fig. 12 B, lower panel). In contrast, treatment of MPTP-injected mice with Juglone showed that Juglone treatment effectively reduced MPTP induced loss of TH positive neurons (Fig. 12 B) and their terminals in striatum (Fig. 12 A). Similarly, by western blot also, we observed attenuation of TH expression in striatum and SN of MPTP treated mice (Fig. 12 C). However, Juglone treatment effectively restored expression of TH in striatum and SN of MPTP treated mice nearly to the control level (Fig. 12 C). To further confirm these findings we measured optical density of striatum (Fig. 12 D) and stereologically counted TH positive neurons in substantia nigra (Fig. 12 E) excluding ventral tegmental area (VTA). We found reduction of striatal TH ODs and significant loss of SNpc TH +ve neurons in MPTP treated mice. However, in MPTP injected mice that also received Juglone showed protection of striatal TH ODs and nigral TH +ve neurons. Collectively, these data suggest that Juglone protects the nigrostriatum against MPTP-toxicity.
Discussion

Here we present novel findings of selective upregulation of Pin1 expression in the dopaminergic neurons of human PD patients and its role in neurodegeneration in a mice MPTP model of PD. First, we show that Pin1 is preferentially upregulated in the MN9D dopaminergic cells and in dopaminergic neurons of nigrostriatum during MPP+/MPTP treatment. Secondly, by employing pharmacological and genetic tools, we establish that Pin1 has a pro-apoptotic role in the dopaminergic neurons. Thirdly, pharmacological inhibitor of Pin1-Juglone attenuates MPP+ / MPTP induced Pin1 expression in MN9D cells / nigrostriatum. Finally, Juglone also rescues mice from behavioral deficits and dopaminergic degeneration in a mice MPTP model of PD.

Pin1 co-localizes with α-synuclein in the Lewy bodies of degenerating neurons of PD and overexpression of Pin1 in a kidney cell line led to Lewy body formation as it increased the half-life of α-synuclein (Ryo et al., 2006). Pin1’s specific enzymatic mechanism targets phosphorylated Ser/Thr-Pro sites and switches a kinase/phosphatase inaccessible Ser/Thr-Pro sites from cis conformation to trans conformation which uncovers substrates for the Proline – directed kinases. Ablation of Pin1 in p53 null mice resulted in accelerated formation of lymphomas and thymic hyperplasia (Takahashi et al., 2007). Proline directed phosphorylation is significant for the regulation of post-mitotic development of neurons and during neurodegeneration (Rudrabhatla and Pant). Growing reports suggest of Pin1’s role in several neurodegenerative disorders and the paucity of information on Pin1’s function during PD led us to this investigation. The roles of Pin1 in Alzheimer’s disease (AD) pathogenesis is a subject of contradiction, while, several investigators report of downregulation of Pin1
during oxidative stress and its association with tautopathy and neurodegeneration during AD (Butterfield et al., 2006; Sultana et al., 2006). On the other hand, inhibition of Pin1 led to attenuation of neurofilament-H phosphorylation and its perikaryal accumulation in AD as well as Amyotrophic Lateral Sclerosis (ALS) models (Kesavapany et al., 2007; Rudrabhatla et al., 2009). Pin1−/− mice exhibited a phenotype of an age dependent accumulation of abnormal and hyperphosphorylated tau and neurodegeneration suggesting a neuroprotective role to Pin1 in AD models (Lu et al., 1999). With the roles of Pin1 largely unclear during neurodegeneration and since Pin1 accumulates in the Lewy body inclusions we sought to understand and characterize Pin1 during PD. We first identified that Pin1 is highly expressed in the pigmented dopaminergic neurons of PD patients in comparison to age-matched controls. Human PD cases are associated with loss of pigmented dopaminergic neurons in SN and our findings of elevated Pin1 expression only in pigmented dopaminergic neurons suggest a possible role of Pin1 in pathogenesis of PD. Our in vitro findings suggest that during treatment of MN9D dopaminergic cells and primary mesencephalic neurons with MPP⁺, there is upregulation of Pin1 at the message and protein levels. Additionally, we identified upregulation of Pin1 expression in nigrostriatum of C57 black mice upon MPTP insult corroborating our in vitro findings. Precedence for upregulation of Pin1 expression in diseased state comes from increased message levels in the circulation of lung cancer patients (Ai et al., 2009), Lymph node metastasis in oral squamous cell carcinoma (Miyashita et al., 2003), hepatocellular carcinomas (Pang et al., 2006), colorectal tumors (Kuramochi et al., 2006). Functionally, heightened levels of Pin1 in cancerous conditions are associated with sensitization of tumor cells to oxidative stress-induced apoptosis. During AD, loss of Pin1 expression is reported to be a mechanism behind neurodegeneration (Akiyama et al., 2005).
On the contrary, upregulation of Pin1 expression during MPTP treatment in the dopaminergic neurons and enhanced expression of Pin1 in human PD cases in our study suggest that the biological/pathological role of Pin1 in PD may be unique.

We show in this study for the first time that Pin1 may also be a possible therapeutic target for PD. Recent studies demonstrated that Pin1 inhibition has anti-cancer effects in experimental studies (Dourlen et al., 2007; Gianni et al., 2009). Juglone (5-hydroxy-1,4-napthalenedione, (C$_{10}$H$_{6}$O$_{3}$) is an aromatic organic napthoquinone, naturally found in the leaves, roots and bark of plants in the black walnut and has been widely used as a Pin1 inhibitor (Esnault et al., 2007; Jeong et al., 2009). Napthoquinone is known to have various physiological activities and also it plays role in induction of apoptosis (Kang et al., 2002). Juglone irreversibly and specifically inactivates enzymatic activity of E. coli parvulin and human Pin1 by modification of thiol groups of cysteine residue in parvulin (Hennig et al., 1998). Juglone plays role in various cellular events such as induction of DNA damage (Wang et al., 2001) and cell death (Paulsen and Ljungman, 2005). Recently, Esnault et al., 2007 showed that Juglone prevented acute and chronic rejection of MHC mismatched, orthotopic rat lung transplants by reducing the expression of IFN-$\gamma$ and CXCL-10 mRNA stability, accumulation and protein expression after cell activation. Juglone also attenuates rheumatoid arthritis development and COX-2 expression in human primary culture RA chondrocytes and CII treated DBA/1J mice (Jeong et al., 2009). Additionally, recent reports suggested anti-inflammatory, anti-viral and anti-fungal properties of Juglone (Omar et al., 2000). Juglone at a dose of 5.7$\mu$M in vitro blocked Pin1’s and E. coli parvulin isomerase activity completely and these doses of Juglone had no effect on other PPIases (Hennig et al., 1998). In our study,
we found 3μM Juglone significantly restored dopamine reuptake in primary mesencephalic neurons and also protected mesencephalic and striatal primary neurons and their neurites from MPP⁺ toxicity (Fig. 4 E). We did not detect any level of toxicity to the neurons with this dose. Additionally, we also demonstrated that Juglone attenuated MPP⁺ induced Pin1 expression in MN9D cells. A recent report used 5mg/kg dose of Juglone to block type-2 rheumatoid arthritis in DBA/1J mice (Jeong et al., 2009). In our study we injected multiple doses of Juglone (3mg/kg) intraperitoneally to the C57BL/6 mice. Importantly, these doses of Juglone were nontoxic. However, 10mg/kg multiple doses of Juglone showed some toxicity to the mice, such as paralysis and 40% death. Here we demonstrate that Juglone effectively attenuates the MPTP-induced expression of Pin1 expression in nigrostriatum. Furthermore, Juglone also restored the behavioral activities and dopamine and its metabolites level in striatum of MPTP treated mice. Additionally, Juglone also protected against MPTP induced loss of TH positive neurons and terminals in the nigrostriatum. Taken together, the observed protective effect of the Pin1 inhibitor Juglone in an MPTP model of PD strongly suggest efficacy of Juglone as a neuroprotective agent at a right dose.

Oxidative stress, neuroinflammation and apoptotic degeneration are the key events underlying PD pathogenesis. The role of Pin1 in the inflammatory responses of PD is not known. Previous reports indicated Pin1’s role in controlling several transcription factors that mediate induction of iNOS, a key inflammatory molecule involved in the pathogenesis of PD (Gothel and Marahiel, 1999; Shen et al., 2005). It has been known that Pin1 binds to the phosphorylated Thr254-Pro residue of transcription factor NF-κB p65 and through this interaction it helps in stabilizing p65 in nucleus, maintaining activation of NF-κB (Ryo et al.,
NF-κB activation is a key inflammatory mediator and (Ghosh et al., 2007) previously reported induction of NF-κB in SN of PD patients and MPTP-treated mice. But it is not known whether there is any interaction between Pin1 and NF-κB in PD. Further research in this area could elucidate the inflammatory roles of Pin1 in pathogenesis of PD. Pin1’s roles during apoptosis is varied. While inhibition of Pin1 led to apoptosis in vascular smooth muscle cells and Pin1 was required for pro-survival signaling in eosinophils (Shen et al., 2009), Pin1’s pro-apoptotic function has been demonstrated during downregulation inhibitor of apoptosis protein-survivin in neuroblastoma cells (Dourlen et al., 2007) and promoting tumor cell death by dissociating tumor suppressor p53 from apoptosis inhibitor iASPP (Mantovani et al., 2007). In nervous system, Pin1 has been shown to regulate oligodendrocyte apoptosis after spinal injury by binding and stabilizing Mcl-1 after JNK3 activation (Li et al., 2007) and also induce death of developing neurons during JNK signaling. Apoptotic signaling of c-Jun N-terminal kinase (JNK) is regulated by Pin1 after JNK activation. Pin1 dissociates from the neuron-specific JNK scaffold protein JIP3, to associate with phosphorylated BH3-only BIM\textsubscript{EL} and activate the mitochondrial apoptotic machinery (Barone et al., 2008). Our findings demonstrate that Pin1 inhibitor Juglone attenuates Parkinsonian toxicant MPP\textsuperscript{+} induced apoptotic events, which are further corroborated with knock-down of Pin1 expression by specific siRNA. Established reports of Pin1’s pro-apoptotic function lends strength to our findings of in a PD model.

In conclusion, we have demonstrated for the first time in our knowledge that Pin1 is upregulated in human PD and MPTP mouse model of Parkinson’s disease. We showed that MPP\textsuperscript{+} induces Pin1 activation in MN9D dopaminergic cell lines and in mesencephalic
primary neuronal cultures. By employing pharmacological and genetic tools to inhibit Pin1, we reported for the first time that Pin1 has a pro-apoptotic role in PD pathogenesis. Additionally, we also demonstrated increased expression levels of Pin1 in SN of human PD brain and MPTP treated mice. By using Pin1 inhibitor Juglone we could effectively attenuate MPTP induced Pin1 expression in mice nigrostriatum. Additionally, Juglone improved behavioral imparities and protected nigrostriatum in MPTP treated mice. These results clearly suggest that Pin1 may serve as a novel pharmacological target for development of neuroprotective agent in PD and Pin1 inhibitor Juglone may be used as novel neuroprotective agent.

References


(MPP+)-induced oxidative stress and apoptosis in mesencephalic dopaminergic neuronal cells. Neurotoxicology 28:988-997.


Zhang D, Anantharam V, Kanthasamy A, Kanthasamy AG (2007) Neuroprotective effect of protein kinase C delta inhibitor rottlerin in cell culture and animal models of
Figure 1. **MPP⁺ induces Pin1 expression.** 

*Panel A*.

Real-time PCR; MN9D cells were treated with 300µM MPP⁺ for 24 hrs and assayed for Pin1 mRNA using qRT-PCR. 

*Panel B*.

Data represent results from six individual measurements and are expressed as mean ± SEM. 

*Panel C*.

Pin1 immunoblot after MPP⁺ treatment. MN9D cells were treated with 300µM MPP⁺ for 12 h and proteins were separated by 15% SDS-PAGE and probed with Pin1 antibody to observe for Pin1 (18kDa) bands. 

*Panel D*.

Quantification of Western blot revealed that the cells have undergone an increase in expression of Pin1 when compared to the control cells. Data represent mean ± SEM from N=3 in three experiments. * P < 0.05, ** P < 0.01 and *** P < 0.001 indicate significant differences between treatment groups as indicated.
Figure 2. Effect of Pin1 inhibitor Juglone on MPP+ induced Pin1 expression, cytotoxicity and caspase-3 activation in MN9D dopaminergic neuronal cells. MN9D cells were treated with 300μM MPP+ for 24 hrs in the presence or absence of 1μM Juglone. 

A, Immunoblotting for Pin1. B, Cytotoxicity of MN9D cells during 300μM MPP+ treatment in the presence or absence of 1μM Juglone. Cytotoxicity was determined by Sytox green
nucleic acid stain by measuring the fluorescence at regular intervals, as described in Materials and Methods. **C**, The two panels demonstrate phase contrast images (left) and fluorescence micrographs (right) to show the extent of Sytox green staining of cells in the field of view. **D**, caspase-3 assay in the presence or absence of 1μM Juglone. Data represent mean ± SEM. of n = 6 from three separate experiments. ***, p < 0.001 as indicated by one-way ANOVA analysis using Dunnett’s multiple comparison tests.
**Figure 3.** siRNA mediated knock down of Pin1 attenuates MPP\(^+\) induced apoptotic events. MN9D dopaminergic neuronal cells were cultured, transfected with Pin1 specific siRNA and exposed to 300µM MPP\(^+\) for 18 to 24 hours. **A.** Immunoblotting for Pin1 from siRNA transfected MN9D cell lysates. **B.** Caspase-3 activation in MN9D cells following MPP\(^+\) treatment. **C.** DNA fragmentation in MN9D cells following MPP\(^+\) treatment. Data represent mean ± SEM of n = 6 from three separate experiments. ****, p < 0.001 as indicated by one-way ANOVA analysis using Dunnett’s multiple comparison tests.
Figure 4.
Figure 4. Juglone inhibits MPP+ induced expression of Pin1 in primary neuronal culture. Mesencephalic and striatal tissues from E15 mouse embryos were cultured and grown on laminin-coated coverslips. The neuronal cultures were treated with 10μM MPP+ for 24 h in the presence or absence of 3μM Juglone. After culture, primary neurons were fixed with 4% paraformaldehyde and incubated with anti-TH and anti-Pin1 antibodies and viewed under a NIKON TE2000 fluorescence microscope. A. Double labeling of TH and Pin1 in primary mesencephalic culture from substantia nigra. B. Double labeling of TH and Pin1 in primary culture from striatum. C & D. Mesencephalic and striatal neuronal process length were quantified using MetaMorph image analysis software, as mentioned in Materials and Methods. E. Dopamine uptake assay from primary striatal culture. Data represent mean ± SEM of n = 6 from three separate experiments. ***, p < 0.001 vs Control or MPP+ as indicated by one-way ANOVA.
Figure 5. Single dose of MPTP administration leads to time dependent increase of Pin1 expression. Mice were administered single dose of MPTP (30 mg / kg) via intraperitoneally and sacrificed at different time points (ranging from 1.5 h to 24 h). A. Expression of Pin1 in mice substantia nigra at different time points after MPTP administration. B. Densitometry analysis of Pin1 obtained from A. C. Expression of Pin1 in mice striatum at different time points after MPTP administration. D. Densitometry analysis of Pin1 obtained from C. E. Pin1 activity assay in SN 6 h after MPTP treatment. Mean density, Pin1/β-actin is plotted on the y-axis. Data represent mean ± SEM of n = 3 from three separate experiments. ***, p < 0.001 vs Control; **, p < 0.01 vs Control; *, p < 0.05 vs Control.
Figure 6.
Figure 6. Juglone attenuates MPTP induced Pin1 message and expression in mice substantia nigra and in striatum. Mice were injected four doses (i.p.) of MPTP (18mg/kg) on a single day and sacrificed at different time points. Another group of MPTP treated mice also received Juglone intraperitoneally at a dose of 3mg/kg 24 h before MPTP injection.  

A. Expression of Pin1 in mice substantia nigra at different time points after MPTP administration.  

B. Densitometry analysis of Pin1 obtained from A. 

C. Expression of Pin1 in mice striatum at different time points after MPTP administration. 

D. Densitometry analysis of Pin1 obtained from C. Twenty four h after MPTP injection, mice were sacrificed and checked Pin1 expression by western blot in substantia nigra (E) and in striatum (G). 

F & H. Densitometry analysis of Pin1 obtained from E & G respectively. 

I. The mRNA expression of Pin1 in SN by quantitative real-time PCR. Mean density, Pin1/β-actin is plotted on the y-axis. Data represent mean ± SEM three separate experiments. ***, p < 0.001 vs control; **, p < 0.01 vs Control or MPTP; * p < 0.05 vs Control or MPTP.
Figure 7

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**Figure 7. Juglone attenuates MPTP induced Pin1 expression in mice substantia nigra.**

Mice were treated with Juglone (3mg/kg) 1 day prior MPTP treatment. Twenty four hr after last dose of MPTP treatment mice were sacrificed and substantia nigra sections were immunolabeled with different antibodies. **A.** DAB immunostaining of Pin1 in substantia nigra. A single enlarged Pin1 positive cell (inset). **B.** Double labeling of global neuronal marker NeuN and Pin1 in substantia nigra. **C.** Mice substantia nigra tissues were immunolabelled for TH (marker of dopaminergic neurons) and Pin1. Higher magnified double labeled pictures of TH and Pin1 (Inset).
Figure 8. Increased expression of Pin1 in human Parkinson’s disease (PD) midbrain. 

A. Human midbrain substantia nigra sections of PD patient and age-matched controls were immunostained for TH (marker of dopaminergic neurons) and Pin1. B. Enlarged image of control section showing decreased co-localization of TH and Pin1 in substantia nigra. C. Neuromelanin containing pigmented TH positive neurons are expressing Pin1 in PD brain.
section. A single, enlarged, pigmented, TH positive dopaminergic neuron shows Pin1 expression (inset) D. Attenuation of Pin1 expression in non-pigmented TH positive neurons in substantia nigra regions PD brain section. A single, enlarged, non-pigmented, TH positive dopaminergic neuron with no Pin1 expression (inset). E. Western blot of Pin1 expression in midbrain of human PD patients.
Figure 9. Expression of Pin1 in mice cortex and hippocampus. Mice were injected four doses (i.p.) of MPTP (18mg/kg) on a single day at 2 h interval. Twenty four h after last dose of MPTP injection mice were sacrificed and cortex and hippocampus regions were dissected out for checking Pin1 expression by Western blot. A. Expression of Pin1 in cortical regions of mice brain. B. Expression of Pin1 in hippocampus regions of mice brain.
Figure 10. Juglone improves motor function in MPTP injected mice. Mice received Juglone (3mg/kg) via i.p. one day prior to MPTP treatment and continued for another 7 days. Five days after MPTP treatment mice were tested for motor functions. A. Moving track of mice using Versaplot software. B. Horizontal activity, C. Vertical activity, D. Total distance travelled (cm), E. Total movement time (s), F. No. of rearing activities and G. Time spent on rotarod. Data are means ± SEM of nine mice per group. *** p < 0.001 vs. control; **, p < 0.01 vs. control; *, p < 0.05 vs. MPTP.
Figure 11. Juglone attenuates MPTP induced loss of dopamine and dopamine-derived metabolites. Mice were treated with Juglone (3mg/kg) via i.p. one day prior MPTP treatment and continued for another 7 days. Seven days after last dose of MPTP treatment mice were sacrificed and A. dopamine, B. DOPAC and C. HVA were measured from striatum by HPLC as mentioned in materials and methods. Data are means ± SEM of six mice per group.

***, p < 0.001 vs. control; **, p < 0.01 vs. control; #, p < 0.01 vs. MPTP.
Figure 12. Effects of Juglone on nigrostriatum in MPTP treated mice. Mice were administered with Juglone (3mg/kg dose) via i.p. one day prior to MPTP injection and continued for 7 days (1 day co-treatment with MPTP and 6 days post-treatment after MPTP). Mice were sacrificed 7 days after MPTP treatment and TH-DAB immunostaining were performed in **A.** striatum (2X magnification), **B.** upper panel - substantia nigra (2X magnification) and lower panel - substantia nigra (10X magnification) **D.** Quantification TH-positive fibers in striatum and **E.** stereological counts of TH-positive neurons in the substantia nigra.
Manuscript Supplementary Figures

Figure 1.

**Figure 1. GABAergic neurons also express Pin1 in striatal primary neuronal culture.** Striatal tissues from E15 mouse embryos were cultured and grown on laminin-coated coverslips. The neuronal cultures were treated with 10µM MPP+ for 24h. After culture, primary neurons were fixed with 4% paraformaldehyde and incubated with anti-GAD and anti-Pin1 antibodies and viewed under a Nikon TE2000 fluorescence microscope. **A.** Double labeling of GAD (marker of GABAergic neurons) and Pin1 in striatal primary neuronal culture. **B.** MPP+ treated striatal neuronal cultures were immunolabelled for TH and GAD.
CHAPTER III

PIN1 MODULATES ACTIVATION OF PROINFLAMMATORY TRANSCRIPTION FACTOR NF-κB IN NEUROINFLAMMATORY PROCESSES IN PARKINSON’S DISEASE

A manuscript to be submitted to The Journal of Immunology

Anamitra Ghosh¹, Dilshan Harischandra¹, Arthi Kanthasamy¹, Vellareddy Anantharam¹, and Anumantha G. Kanthasamy¹

¹Department of Biomedical Sciences, Iowa Center for Advanced Neurotoxicology, Iowa State University, Ames, IA

Abstract:

Glial activation and neuroinflammation contribute to the progressive degeneration in Parkinson’s disease (PD). Nuclear factor kappa beta (NF-κB), an important modulator of inflammation gets activated in PD brain and MPTP-treated mice brain and regulates cell proliferation, inflammation, and apoptosis. The prolyl isomerase, Pin1 plays important pathophysiological roles in cancers and Alzheimer’s disease where it directly binds to the NF-κB protein, p65 and regulates NF-κB signaling. We now present evidences that interaction of Pin1 and p65 occurs in mouse microglial cells and controls the inflammatory processes. We demonstrated that Pin1 and p65 protein levels are enhanced in BV2 and
primary microglial cells in response to different stimuli. Paradoxically, Juglone, the pharmacological inhibitor of Pin1, attenuated inflammatory stimuli induced Pin1 and p65 upregulation. By attenuating p65 activation, Juglone also reduced nitrite production and cytokine release from microglial cells in response to different stimuli. In the MPTP mouse model of PD, an increased expression of nigral Pin1 in microglial and astroglial cells were observed. Furthermore, the Pin1 inhibitor Juglone attenuated p65 dependent expression of proinflammatory cytokines and inducible nitric oxide synthase (iNOS) in MPTP-treated midbrain, suggesting a proinflammatory role of Pin1. Taken together, these results suggest that Pin1 is an important regulator of NF-κB mediated microglial activation and neuroinflammatory processes and therapeutic targeting of Pin1 could be used as a therapeutic target in the future.

**Introduction:**

Parkinson’s disease (PD) is one of the most common neurodegenerative motor disorders and pathologically is characterized by the selective loss of dopaminergic neurons in the substantia nigra of ventral mid brain and their terminal in striatum, resulting in loss of striatal dopamine and appearance of cytoplasmic inclusions known as Lewy bodies in surviving neurons of substantia nigra (SN) (Dunnett and Bjorklund, 1999; Dauer and Przedborski, 2003). PD is chronic and progressive in nature and characterized predominantly by resting tremors, muscular rigidity, bradykinesia and postural instability along with several non-motor symptoms (Savitt et al., 2006). The etiology of PD is not known completely, but recent evidences suggest that glial cells mediated inflammation and oxidative stress increased the risk of developing PD (Dauer and Przedborski, 2003). Continuous and prolonged
microglial and astroglial activation in close proximity of degenerating dopaminergic neurons in the substantia nigra (SN) is evident in PD patients and animal models of the disease (McGeer et al., 1988; Hirsch and Hunot, 2009). Various in vitro and in vivo animal models have demonstrated elevation of the key enzymes involved in the production of reactive oxygen species (ROS) or reactive nitrogen species (RNS), such as microglial NADPH oxidase and inducible nitric oxide synthase (iNOS) in SN (Gao et al., 2003; Wu et al., 2003). In addition to this, a variety of pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-α), interleukin (IL) -1beta (β), IL-6, interferon gamma (IFN-γ) and other immune cytokines are found either in CSF or affected regions of PD brain (Nagatsu et al., 2000). However, the mechanism behind activation of glial cells and its role in the progressive dopaminergic neurodegeneration remains elusive. However, microglial NADPH oxidase which releases superoxide in the SN could contribute to the oxidative stress mediated dopaminergic neurodegeneration (Wu et al., 2002). Also, the activation of NF-κB in microglial and astroglial cells play critical neurotoxic role by mediating proinflammatory cytokine levels in the SN (Ghosh et al., 2007).

Reversible phosphorylation on Ser/Thr – Pro (S/T-P) motifs regulated by Pro-directed protein kinases and phosphatases is an important molecular switch in controlling various cellular processes (Jager et al., 2006). The protein interacting with never in mitosis – A (Pin1) is an 18kDa isomerase enzyme and it binds to and cis/trans isomerizes a specific subset of serine-proline or threonine-proline bonds (Ser/Thr-Pro) in its target proteins (Lu et al., 2007). Deregulation of Pin1 signaling is implicated in a growing number of pathological conditions such as Alzheimer’s disease (AD) (Maruszak et al., 2009), asthma (Esnault et al., 2008), Pick disease (Ramakrishnan et al., 2003) and different cancers (Ryo et al., 2002; Li et
al., 2006; Pang et al., 2006). Pin1 interacts with mitochondrial – BH3-only protein BIM<sub>EL</sub> and activates c-JUN to regulate a neuron-specific activation of mitochondrial apoptotic machinery during neurodegeneration (Becker and Bonni, 2007). Recently Ryo et al., reported that Pin1 overexpression facilitates formation of α-synuclein inclusions in a cellular model of α-synuclein aggregation and Pin1 also localizes in Lewy bodies in PD patients (Ryo et al., 2006).

Among 50 Pin1 target proteins, NF-κB p65 is one of them (Ryo et al., 2003). Promoter regions of proinflammatory molecules like iNOS and proinflammatory cytokines (IL-1β, IL-6, TNF-α) contain the DNA binding site for NF-κB (Ghosh and Karin, 2002). Furthermore, the inhibition of NF-κB activation reduces the induction of proinflammatory molecules (Ghosh and Karin, 2002). As, Pin1 is a positive regulator for NF-κB controlled promoters (Ryo et al., 2003), Pin1 plays an important role in modulating NF-κB regulated inflammatory processes. In an unstimulated state, NF-κB protein complex exists in cytoplasm bound to an inhibitory family of proteins called IkB. During activation stimuli, IkB (IKK) kinase complex gets activated (Ghosh and Karin, 2002). This complex then phosphorylates and degrades IkB by the ubiquitin-proteasome pathway. Free NF-κB protein complex then translocate to the nucleus, where they active the proinflammatory genes (Karin, 2006).

Here, in our study we demonstrated that Pin1 and NF-κB p65 activation are induced in BV2 and primary microglal cells in response to different stimuli and Pin1 strongly associates with p65. Juglone, a pharmacological inhibitor of Pin1, attenuated both Pin1 and p65 expression in microglial cells. NF-κB mediated inflammatory processes were also
inhibited by Juglone. In addition to that, MPTP-treated mice showed upregulation of Pin1 in nigral microglial and astrocytes. Moreover, treatment of MPTP-injected mice with Juglone resulted in attenuation of inflammatory reactions.

Materials and methods:

Chemicals and biological reagents. 1-Methyl-4-phenyltetrahydropteridine (MPP⁺ iodide) and MPTP-HCl were purchased from Sigma (St. Louis, MO, USA). Pin1 inhibitor Juglone was purchased from Calbiochem. RPMI, neurobasal medium, B27 supplement, fetal bovine serum, L-glutamine, Sytox assay dye, IR-dye tagged secondary antibodies, penicillin, and streptomycin and other cell culture reagents were purchased from Invitrogen (Gaithersburg, MD). Recombinant murine TNF, LPS (E. coli 0111:B4) and cytosine arabinoside were purchased from Sigma-Aldrich (St. Louis, MO).

Treatment paradigm. MPP⁺ (10μM) was added to the cells for the duration of the experiment. The cells were detached from the flask using a rubber policeman and centrifuged at 200 x g for 5 min, washed with ice-cold PBS twice and homogenized with Radio-immunoprecipitation Assay (RIPA) buffer. Cell lysates, collected by spinning down the cell fragments at 20,000 x g for 45 min at 4°C, were used to determine changes to Pin1 expression.

qRT-PCR. After treatment, total RNA were extracted from the mice brain SN using Absolutely RNA Miniprep Kit (Agilent Technologies, Santa Clara, CA) following the manufacturer’s protocol. Total RNA was treated with DNase I to remove DNA contamination and then reversibly transcribed into first-strand cDNA using the SuperScript III first-strand synthesis system (Invitrogen, Carlsbad, CA) as described in the kit.
instructions. SYBR-green Quantitative PCR was performed with validated primers of TNF-α, IL-1β, iNOS, IL-6 and control 18S primers (Qiagen, Valencia, CA) using RT² SYBR® Green qPCR Master Mix (SABiosciences, Fredrick, MD) for the mice tissue.

**Sytox cell death assay.** The BV2 cells were incubated with 10ng/ml TNF-α for 18 h in the presence or absence of different doses of Juglone and cell death was determined by the cell impermeable Sytox green (Molecular Probes, Eugene, OR). Sytox green intercalates with the DNA in the membrane compromised cells to produce a green fluorescence that is quantifiable at excitation 485 nm and emission 538 nm using the fluorescence microplate system (Synergy 4, Biotek, Winooski, VT) and also allows to be viewed under fluorescence microscope.

**Microglial nitric oxide detection.** Nitric oxide production by BV2 cells was measured indirectly by quantification of nitrite in the supernatant using the Griess reagent (Sigma Aldrich). BV2 microglial cells were plated in poly-D-lysine coated 96-well plates at 50,000 cells per well. Cells were treated with either 1µg/ml LPS for 24 h or 10ng/ml TNF-α for 18 h in presence or absence of different doses of Juglone and 100 µL of supernatant was collected from each well and an equal volume of the Griess reagent was added. The samples were incubated on a plate shaker at room temperature for 15 min until a stable color was obtained. The absorbance at 540 nm was measured using a Synergy 2 multi-mode microplate reader (BioTek Instruments) and the nitrite concentration determined from a sodium nitrite standard curve.

**Multiplex cytokine and chemokine Luminex immunoassays.** BV2 and primary microglia from time pregnant pups from PKCδ wild-type mice were obtained and seeded in poly-D-lysine coated 96-well plates respectively. The cells were treated for 24 h with 1µg/ml LPS
(for BV2) or 100 ng/mL LPS (for p. microglia) in presence or absence of 3µM Juglone. After treatment, 50 µL of supernatant from each well was collected and frozen at -80°C. The levels of cytokines and chemokines in the supernatants were determined using the Luminex bead-based immunoassay platform (Vignali, 2000) using pre-validated multiplex kits (Milliplex mouse cytokine and chemokine panels – Millipore Corporation) according to the manufacturer’s instructions.

**Intracellular reactive oxygen species (iROS) detection.** Intracellular reactive oxygen species (iROS) were determined using the fluorescent probe 2’,7’-dichlorodihydrofluorescein diacetate (DCFH-DA) (Calbiochem), according to previously published reports (Zhang et al., 2005; Qian et al., 2007). Magnetically separated microglia from PKCδ wild-type mice were plated at 10⁵ cells per well in 96-well plates and allowed to attach overnight. Before treatment, cells were washed in HBSS and incubated with 40 µM DCFH-DA in HBSS containing 2% FBS for 30 min. Cells were then treated with 100 ng/mL LPS for 24h in the presence or absence of 3µM Juglone. The fluorescence intensity was determined using a Synergy-2 multi-mode microplate reader at an excitation of 485/20 nm and an emission of 530/25 nm. The fluorescence value from the control cultures was subtracted as background and the increase in fluorescence with treatments was expressed as increased iROS as previously described (Zhang et al., 2005; Qian et al., 2007).

**Animals and MPTP treatment.** Eight- to 12-week-old male C57BL/6 mice weighing 24 to 28 g were housed in standard conditions: constant temperature (22 ± 1°C), humidity (relative, 30%), and a 12-h light/dark cycle. Mice were allowed free access to food and water. Use of the animals and protocol procedures were approved and supervised by the Committee on Animal Care at Iowa State University (Ames, IA, USA). Mice received four injections of
MPTP-HCl (15mg/kg of free base; Sigma-Aldrich) in 1XPBS at 2 h intervals via i.p. The control mice received only 1XPBS.

**Juglone treatment in mice.** Juglone was dissolved in 10% ethanol and was administered to mice via intraperitoneal injection. Based on other reports, (Esnault et al., 2007b; Jeong et al., 2009) we injected 10 mg / kg dose of Juglone intraperitoneally. However, we observed nearly 40% mortality rate of mice. Hence, we chose a lower dose (3 mg / kg) of Juglone and we never noticed any death or other behavioral imparities in mice. First dose of Juglone (3 mg / kg) was administered 24 h before 1st dose of MPTP injection, second dose of Juglone was administered 3 h before the 1st dose of MPTP injection and third dose of Juglone was administered 3 h after the last dose of MPTP injection. Mice were sacrificed 24 h after the last dose of MPTP.

**Immunoblotting.** After treatment, cells and mice brain tissues were collected and resuspended in modified RIPA buffer containing protease and phosphatases inhibitor cocktail. Cell suspensions were sonicated after resuspension, while tissues were homogenized, sonicated and then centrifuged at 14000 x g for 1 hr at 4°C. Lysates were separated on 8-12% SDS – polyacrylamide gel electrophoresis (PAGE). After the separation, proteins were transferred to a nitrocellulose membrane, and nonspecific binding sites were blocked by treating with Odyssey® blocking buffer (LiCor, Lincoln, NE). Pin1 (1:2000; mouse monoclonal; R&D Systems, Minneapolis, MN), p65 (1:500, rabbit polyclonal; Santa Cruz, CA), β-Actin (1:5000; mouse monoclonal; Sigma, St. Louis, MO), iNOS (1:350, rabbit polyclonal; Santa Cruz, CA) were used to blot the membranes. Secondary IR-680-conjugated anti-mouse (1:10000; Molecular Probes, Carlsbad, CA) and IR dye 800 anti-rabbit (1:10000;
Rockland) secondary antibodies were used and scanned in Odyssey IR imaging system (LiCor, Lincoln, NE).

**Immunoprecipitation.** For immunoprecipitation studies, briefly, cells were lysed in immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 1% Triton X-100, 1 X halt protease inhibitor mixtures), and the resultant lysates were incubated on ice for 15 min followed by centrifugation at 16,000 X g for 15 min. The supernatant fractions were then precleared with protein A or protein G beads for 30 min at 4°C followed by centrifugation at 16,000 g at 4°C for 10 min. Five micrograms of the indicated antibody along with 50 XI of 50% of protein A or protein G beads were added to the cell lysates and incubated overnight at 4°C on a rotator. The immunoprecipitates were collected, washed extensively with cold PBS, and prepared for SDS-PAGE gel by addition of 2% SDS sample buffer and then boiling for 10 min (Jin et al.).

**Mesencephalic Primary Neuron Cultures and Treatment.** Primary mesencephalic and striatal neuronal cultures were prepared from the ventral mesencephalon of gestational 14- to 15-day-old mice embryos as described previously (Zhang et al., 2007). Briefly, mesencephalic tissues from E14 to 15 mouse embryos were dissected and maintained in ice-cold calcium-free Hanks’ balanced salt solution and then dissociated in Hanks’ balanced salt solution containing trypsin-0.25% EDTA for 20 min at 37°C. The dissociated cells were then plated at equal density of 0.1 million cells per well on 12-mm coverslips precoated with 0.1 mg/ml poly-D-lysine. Cultures were maintained in neurobasal medium fortified with B-27 supplement, 500 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA). The cells were maintained in a humidified CO₂ incubator (5% CO₂ and 37°C) for 24 h. Half of the culture medium was replaced every 2 days.
Approximately 6- to 7-day-old cultures were used for experiments. Primary mesencephalic and striatal dopaminergic neuronal cells were exposed to 10 µM MPP⁺ in the presence or absence of Juglone (1 µM) for 24 h.

**Immunocytochemistry.** The primary mesencephalic and mixed neuro-glia cultures were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 20 minutes and processed for immunocytochemical staining as described previously (Ghosh et al., 2010). First, nonspecific sites were blocked with 2% bovine serum albumin, 0.5 % Triton X-100 and 0.05% Tween-20 in PBS for 45 min at room temperature. Cells were then incubated with different primary antibodies such as TH (1:1600, mouse monoclonal; Millipore, Billerica, MA), IBA-1 (1:1000, goal polyclonal; Chamicon), GFAP (1:1000; mouse monoclonal; Chemicon), Pin1 (1:400, goat polyclonal; Santa Cruz) at 4°C overnight. Appropriate secondary antibodies (Alexa Fluor 488 or 594 or 555; Invitrogen) were used followed by incubation with 10µg/ml Hoechst 33342 (Invitrogen) for 5 min at room temperature to stain the nucleus. The coverslip containing stained cells were washed twice with PBS and mounted on poly-D lysine coated slides (Sigma, St Louis). Cells were viewed under a NIKON inverted fluorescence microscope (model TE-2000U; NIKON, Tokyo, Japan) and images were captured with a SPOT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

**Immunohistochemistry.** Twenty four hr. after MPTP treatment, mice were perfused with 4% paraformaldehyde and post fixed with PFA and 30% sucrose respectively as described previously (Ghosh et al., 2009). Next, 30 µm coronal SN free-floating sections were blocked with 2% bovine serum albumin, 0.5 % Triton X-100 and 0.05% Tween-20 in PBS for 1 h at room temperature. Sections were incubated with different primary antibodies such as anti-
Pin1 (1:500; goat or rabbit polyclonal), anti-IBA-1 (1:750; goat polyclonal), anti-TH (1:1600; mouse monoclonal; Chemicon, Temecula, CA), anti-GFAP (1:1000; Chemicon) for overnight at 4°C. After washing with PBS, sections were incubated in appropriate secondary antibodies (Alexa Fluor 488 or 594 or 555 from Invitrogen) for 2 h. followed by incubation with 10µg/ml Hoechst 33342 for 5 min at room temperature to stain the nucleus. Sections were viewed under a NIKON inverted fluorescence microscope (model TE-2000U; NIKON, Tokyo, Japan); images were captured with a SPOT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) using MetaMorph software, version 5.0 (Molecular Devices, Sunnyvale, CA).

**Statistical analysis.** Data were analyzed with Prism 3.0 software (GraphPad Software, San Diego, CA). Bonferroni and Dunett multiple comparison testing were used. Differences with $P < 0.05$ were considered significant.

**Results:**

**Pin1 protein expression is enhanced in BV2 cells in response to different stimuli**

Accumulation of Pin1 in the Lewy bodies of PD and promotion of $\alpha$-synuclein aggregates during over-expression of Pin1 in a kidney cell line has left a lot of unanswered questions about the role of Pin1 during PD (Ryo et al., 2006). Very little has been known about the role of Pin1 in inflammatory reactions. As glial activation and sustained nigral neuroinflammation are evident in PD patients, we wanted to check the expression of Pin1 in BV2 microglial glial cells in the presence or absence of different inflammatory stimuli. BV2 microglial cells were treated with 1µg/ml LPS for 24h and after treatment cells were collected and immunoblotted for checking expression of Pin1. We found increased expression of Pin1 in lysates of LPS treated BV2 cells with statistically significant difference
compared with the control BV2 cells (p < 0.01) (Fig. 1A-B). Next, we wanted to see whether parkinsonian toxicant MPP⁺ could also induce Pin1 expression in BV2 cells. Cells were treated with 10µM MPP⁺ for 24h and checked for Pin1 expression. Significant induction of Pin1 expression was observed in MPP+ treated BV2 cells compared to control BV2 cells (p < 0.01) (Fig. 1C-D). Further, TNF-α also induced dose dependent induction of Pin1 protein levels in BV2 cells, with highest induction at 10ng/ml dose. (Fig. 1E-F). Together, these results demonstrate for the first time that protein levels of Pin1 upregulated in response to different stimuli in activated microglial cells.

Parkinsonian toxicant MPP⁺ induces Pin1 expression in primary neuro-glial mixed culture

After establishing the induction of Pin1 protein expression in activated BV2 cells, we studied the subcellular localization of this isomerase in primary mesencephalic mixed neuro-glial culture. Neuron-glial mix cultures were obtained from day 14 time pregnant C57BL/6 female mice embryos. The mixed neuro-glial cultures were treated with 10µM MPP⁺ for 24 h. After treatment, cells were fixed with 4% paraformaldehyde and immunostained either with IBA-1(marker of microglia) and Pin1 (Fig. 2A) or GFAP (marker of astrocytes) and Pin1 (Fig. 2B). We used Hoechst to stain the nucleus. In unstimulated mixed neuro-glial culture, we observed very little staining of Pin1 in IBA-1 positive microglial cells and GFAP positive astrocytes. However, stimulation with MPP⁺ resulted an increased staining of Pin1 in the cytosol of microglial and astrocytes (Fig 2A-B). In addition to that we also checked Pin1 expression in primary astrocytes separated from day 1 old pups. MPP⁺ treatment causes robust upregulation of Pin1 protein expression compared to unstimulated control astroglial
cells (Fig. 2C-D). Together, these results suggest that Pin1 is a cytosolic protein whose level goes up in glial cells in response to MPP⁺.

**Activation of p65 in BV2 cells in response to different inflammatory stimuli**

The promoter region of pro-inflammatory molecules contains the DNA binding site for NF-κB and inhibition of NF-κB reduces the induction of proinflammatory molecules (Hayden MS et al., 2004). A recent study reported MPP⁺ induced activation of p65 in mouse BV2 microglial cells (Ghosh et al., 2007) and we sought to confirm this finding. BV2 microglial cells were treated with 10µM MPP⁺ for 24h. After treatment, cells were collected and immunoblotted for checking Pin1 expression (Fig. 3A). We found increased expression of p65 in MPP⁺ treated cells. Similar with MPP⁺ treatment, cells were also treated with LPS (1µg/ml) for 24h and checked for Pin1 expression. LPS treatment resulted in robust increase in p65 protein expression in BV2 cells compared to untreated control cells (Fig. 3B). Further, TNF-α dose dependently increased p65 expression in BV2 cells, with maximum induction at 10ng/ml dose. After establishing the maximum induction of p65 with 10ng/ml TNF-α dose, we were interested to see at what time point after TNF-α treatment, p65 expression goes up maximum. BV2 cells were treated with 10ng/ml TNF-α for different time points ranging from 6h to 24. We found maximum induction of p65 expression at 18h of TNF-α treatment, with some upregulation at 24h time point.

**Pin1 binds to p65 in BV2 microglial cells**

To determine if a p65-Pin1 interaction occurs within BV2 cell, co-immunoprecipitation experiments were performed. BV2 cells were treated with either MPP⁺ (10um) for 24h or 1µg/ml LPS for 24 h or TNF-α (10ng/ml) for 18h. Immunoprecipitation
was then carried out using a p65 antibody, followed by immunoblotting for for Pin1 and p65 (Fig. 4A). Endogeneous p65 and Pin1 show some association in the control lanes (unstimulated state), which is enhanced with MPP⁺ or LPS or TNF-α. To demonstrate the specificity of this interaction, we did reversed immunoprecipitation (Fig. 4B). Here also, some association between Pin1 and p65 could be noticed in control lanes. However, in MPP⁺ or LPS or TNF treated cells increased association between Pin1 and p65 occurred (Fig. 4B). These results clearly say that there is Pin1 and p65 interaction in mouse microglial BV2 cells.

**Juglone inhibits Pin1 expression in BV2 cells**

After establishing induction of Pin1 in BV2 cells in response to different stimuli, we were interested to see whether pharmacological inhibitor of Pin1 – Juglone could attenuate the Pin1 expression in BV2 cells. To optimize the dose of Juglone, we measured cytotoxicity of BV2 cells using a live-cell impermeable fluorescent dye – Sytox green during treatment of TNF-α in the presence or absence of Pin1 inhibitor – Juglone (Fig 5A-B). As expected, 10ng/ml TNF-α didn’t cause any toxicity to the BV2 microglial cells. Co-treatment with Juglone show some dose dependent cytotoxicity (Fig 5A-B), with significantly high toxicity at 10μM (p < 0.01) and 20μM (p < 0.001) doses of Juglone, where as 1μM and 3μM doses didn’t had any significant toxicity. Next, BV2 microglial cells were treated with 10μM MPP⁺ for 24h in the presence or absence of 3 different doses of Juglone (1μM, 3μM and 10μM). We observed a dose dependent inhibition of MPP⁺ induced Pin1 expression with Juglone treatment, with highest inhibition at 3μM dose (Fig. 5C). 3μM Juglone also attenuated LPS and TNF-α -induced Pin1 expression in BV2 cells (Fig. 5 D-E). To further confirm, our findings in western blotting, we did immunocytochemistry in primary mesencephalic mixed
neuro-glial culture. Neuron-glial mix cultures were obtained from day 14 time pregnant C57BL/6 female mice. The mixed neuro-glial cultures were treated with 10µM MPP⁺ in the presence or absence of 3µM Juglone for 24 h. After treatment, cells were fixed with 4% paraformaldehyde and immunostained either with IBA-1 (marker of microglia) and Pin1 or GFAP (marker of astrocytes) and Pin1 (Fig. 5F). We used Hoechst to stain the nucleus. As expected, MPP⁺ treatment resulted in increased expression of Pin1 in the cytosol of microglial and astrocytes. However, 3µM Juglone treatment attenuates MPP⁺ induced induction of Pin1 in microglia and astrocytes.

**Juglone inhibits p65 activation in activated BV2 cells**

After establishing an inhibitory role of Juglone in Pin1 expression, we were curious to see whether Juglone had any effect on p65 activation. BV2 cells treated with TNF-α (10ng/ml) in the presence or absence of Juglone (3µM) for 18h, were immunostained for Pin1 and p65 double labeling. As expected, TNF-α resulted in increased expression of total p65 in the nucleus (Fig. 6A). However, Juglone treatment attenuated p65 translocation to the nucleus, as very few microglial cells were found with localization of p65 in the nucleus. To confirm these immunofluorescence findings, we did western blotting of p65 in BV2 cells. Cells were treated with MPP⁺ in the presence or absence of 3µM Juglone for 24h. As expected, MPP⁺ treatment resulted in increased expression of p65 expression whereas 1µM and 3µM doses of Juglone dose dependently inhibited p65 expression (Fig. 6B-C). Similar to that, LPS and TNF-α also caused an increase of p65 expression in BV2 cells. However, 3µM Juglone treatment significantly attenuated p65 expression both in LPS and TNF-α treated BV2 cells (Fig. 6C-F).

**Juglone attenuates LPS and TNF-α induced nitric oxide production in microglial cells**
Nitric oxide production and intracellular ROS (iROS) generation are important aspects of the microglial activation response and are particularly relevant in PD models where deregulation of microglial activation is believed to augment dopaminergic neurodegeneration via oxidative stress. We checked if microglial nitric oxide generation is attenuated in the presence of Pin1 inhibitor Juglone. Nitric oxide production was measured indirectly using Griess assay both in BV2 cells and primary microglia (Fig 7A-C). BV2 cells were treated with LPS (300ng/ml) in the presence or absence of Juglone (3µM). LPS treatment for 24 h induced a sharp increase in nitrite levels (> 1.5µM) in BV2 cells, while Juglone dose dependently attenuated LPS-induced nitrite levels with maximum attenuation at 3µM dose of Juglone (Fig. 7A). Similar to that, 3 µM Juglone also significantly (p < 0.05), inhibited LPS-induced nitrite levels in primary microglial cells from day 1 old pups (Fig. 7C). TNF-α also induced significant increase in nitrite release compared to untreated BV2 cells (p < 0.001) (Fig. 7B). However, Juglone treatment dose dependently attenuated TNF-α induced nitrite levels in BV2 cells. Next, we wanted to test whether Juglone inhibited iNOS expression in BV2 cells. Cells were treated with 10µM MPP+ in presence or absence of 3µM Juglone for 24h. Western blot analysis revealed that Juglone attenuated MPP+ induced iNOS protein expression (P < 0.05) (Fig. 7D). Similarly, Juglone also reduced LPS or TNF-α induced iNOS expression in BV2 cells (Fig. 7E-F). Together, these results support that inhibition of Pin1 could lead to attenuation of microglial inflammatory reactions.

**Juglone attenuates microglial cytokines in response to LPS stimulation**

To further establish the role of Pin1 in regulating the microglial activation response, we used BV2 cells and primary microglial cultures from day 1 old pups. BV2 cells were treated with 300ng/ml LPS in presence or absence of 3µM Juglone. 24 h after treatment, the
levels of various pro-inflammatory cytokines in the supernatant were determined using a multiplex bead-based Luminex assay system (Milipore Bioplex). In LPS-treated BV2 cells, the levels of IL-6 (p < 0.001), TNF-α (p < 0.001) and IL-1β (p < 0.001) increased several folds compared to untreated cells. However, Juglone significantly reduced LPS induced IL-6 (p < 0.001), TNF-α (p < 0.01) and IL-1β (p < 0.001) release from BV2 cells (Fig. 8A-C). Similarly, we also measured cytokine response in primary microglia treated with LPS and Juglone. LPS triggered robust increase in IL-6, TNF-α and IL-1β release in primary microglia cells, while Juglone significantly attenuated LPS-induced release of proinflammatory cytokines (Fig. 7D-F). These further indirectly substantiate a role of Pin1 in modulating inflammatory reactions.

**Expression of Pin1 in glial cells of MPTP-treated mouse**

After establishing the possible role of Pin1 in induction of inflammatory reactions in vitro, we examined whether the activation of Pin1 was induced in midbrain of MPTP mouse model of PD. Mice were treated with 4 doses of MPTP at 18mg/kg/dose via i.p. at 2 h interval. 24h after treatment, mice were sacrificed and frozen substantia nigra (SN) sections were processed for immunostaining. MPTP treatment led to robust increase in Pin1 expression (red color) in the SN compared to saline treated control mice (Fig. 9). Double-label immunofluorescence analysis indicates that Pin1 was principally expressed by astrocytes (Fig. 9A) microglia (Fig. 9B). This Pin1 was present in cytoplasm because it did not colocalize with DAPI (Fig. 9).

**Activation of p65 in nigrostriatum of MPTP-treated mice and effect of Juglone on MPTP-induced inflammation in midbrain**
A previous report shows selective activation of NF-κB in substantia nigra of MPTP-treated mouse led to increased inflammatory reactions (Ghosh et al., 2007). We also found activation of p65 in BV2 microglial cells and association of p65 and Pin1 in response to different stimuli (Fig. 2-4). To validate our results obtained with BV2 cells, we first checked protein expression of p65 in nigrostriatum of mice. Acute MPTP treatment led to increased expression of p65 both in SN and striatum in time dependent manner with highest expression at 24h treatment (Fig. 10A-B). Promoter regions of proinflammatory molecules contain the DNA binding site for NF-κB (Hayden and Ghosh, 2004). Because, Pin1 inhibitor Juglone inhibited the activation of p65 in vitro in the microglial cells in response to different stimuli (Fig. 6), we examined whether Juglone was able to suppress the expression of various proinflammatory molecules and cytokines in the midbrain. As shown by quantitative real-time PCR (Fig. 10C) experiments, MPTP treatment led to marked increase in mRNA expression of TNF-α , IL-1β, IL-6 and iNOS in the ventral midbrain. However, 3mg/kg Juglone significantly inhibited MPTP-induced expression of TNF-α (p < 0.05), IL-1β (p < 0.05) and iNOS (p < 0.05) (Fig 10C). But, expression of IL-6 was not significantly attenuated in MPTP treated mice that also received Juglone. Immunofluorescence analysis of iNOS also showed increased expression of nigral iNOS protein expression in MPTP treated mice compared to PBS treated control mice and that iNOS protein was expressed by GFAP-positive astrocytes (Fig. 11A) and IBA-1 positive microglia (Fig. 11B). Inset pictures clearly showed colocalized glial cells and iNOS (merged color of yellow from green and red). However, Juglone suppressed MPTP-induced iNOS expression in astrocytes and microglial cells (Fig. 11A & B), as very few colocalized cells were observed in SN. These results clearly demonstrate that inhibition of Pin1 could be anti-inflammatory.
Discussion:

Activation of microglia and astrocytes have been well documented in post mortem PD brains and mouse models of PD. Activated microglia-induced inflammatory reactions can be both sustained and progressive (Gao et al., 2003; McGeer et al., 2003), which means microglial activation leads to neurodegeneration and neuronal injury, which in turn further reactives microglial activation. But, the signaling pathways regulating microglial activation remains unclear. A recent study shows that proinflammatory transcription factor NF-κB regulates microglial and astroglial proinflammatory responses in the substantia nigra and selective inhibition of NF-κB attenuates inflammatory reactions (Ghosh et al., 2007). NF-κB regulates proinflammatory genes such as TNF-α, IL-1β, Il-6 and iNOS in animal and human studies (Bremner and Heinrich, 2002). In our study, we have shown increased expression of Pin1 and p65 and translocation of p65 to nucleus in microglial cells following stimulation with MPP⁺ or LPS or TNF-α. We also reported association between Pin1 and p65 following MPP⁺ or LPS or TNF-α induction. Earlier, Ryo et al., 2003 demonstrated that Pin1 binds to the phosphorylated Thr²⁵⁴-Pro of p65 and subsequently inhibited IkBa binding (Ryo et al., 2003). Thus, NF-κB activation along with increased Pin1 expression in response to MPP⁺ or LPS or TNF-α treatment is associated with the increased production of proinflammatory molecules.

Our results in this study indentify Pin1 as a novel intracellular protein regulating the glial activation both in vitro and in MPTP mouse model of PD. We demonstrated that Pin1 protein level is induced during microglial activation by distinct mechanisms and stimuli including TNF-α, MPP⁺and LPS. Our results showed increase in nitric oxide production in BV2 and primary microglial cells demonstrated that Pin1 can regulate the microglial RNS
generation machinery, which plays vital role in dopaminergic neurodegeneration. In addition to that, multiplex cytokine assays further demonstrated the involvement of Pin1 controlled NF-κB activation in microglial activation response.

Pin1 is an 18kDa enzyme with two domains. N-terminal amino acids 1-19 comprised the WW domain which determines substrate specificity, whereas the C-terminus contains the catalytic PPIase (peptidyl-prolyl isomerase) domain (Yeh and Means, 2007). Significant higher levels of Pin1 protein is expressed in many human cancers like liver, breast the prostate cancers (Wulf et al., 2001; Bao et al., 2004; Pang et al., 2006). Growing reports suggest Pin1’s role in several neurodegenerative disorders and the lack of information on Pin1’s function during inflammation mediated PD led us to this investigation. The roles of Pin1 in Alzheimer’s disease (AD) pathogenesis is not clear enough, while, several investigators reported downregulation of Pin1 during oxidative stress and its association with tauopathy and neurodegeneration during AD (Butterfield et al., 2006). Some other researchers demonstrated that inhibition of Pin1 led to the attenuation of neurofilament-H phosphorylation and its perikaryal accumulation in AD as well as Amyotropic Lateral Sclerosis (ALS) models (Kesavapany et al., 2007; Rudrabhatla et al., 2009). Pin1−/− mice exhibited a phenotype of an age dependent accumulation of abnormal and hyperphosphorylated tau and neurodegeneration suggesting a neuroprotective role of Pin1 in AD models (Lu et al., 1999). The role of Pin1 in the inflammatory processes of PD is not clear enough. Pin1 controls expression of several transcription factors that mediate induction of iNOS, a key inflammatory molecule involved in the pathogenesis of PD (Gothel and Marahiel, 1999). While inhibition of Pin1 led to apoptosis in vascular smooth muscle cells and Pin1 was required for pro-survival signaling in eosinophils (Shen et al., 2009), Pin1’s
pro-apoptotic function has been demonstrated during downregulation inhibi\n\ntn of apoptosis protein-survivin in neuroblastoma cells (Dourlen et al., 2007) and promoting tumor cell death by dissociating tumor suppressor p53 from apoptosis inhibitor iASPP (Mantovani et al., 2007). In the nervous system, Pin1 has been shown to regulate oligodendrocyte apoptosis after spinal injury by binding and stabilizing Mcl-1 after JNK3 activation (Li et al., 2007) and also induce death of developing neurons during JNK signaling. Since Pin1 role is not clear during neurodegeneration and as it accumulates in the Lewy body inclusions we wanted to understand role of Pin1 in inflammation processes during PD. We first identified that Pin1 is highly expressed in the BV2 cells and primary glial cells (both microglia and astrocytes) in response to different stimuli.

There are several microglial activating agents that causes neurotoxicity. These agents include LPS, TNF-α and MPP⁺. LPS is a Gram-negative bacterial endotoxin that causes microglial cell activation (Kim et al., 2000). LPS binds to an LPS receptor membrane complex on microglial cells and activates nuclear localization of transcription factor nuclear factor κB (NF-κB ) and subsequent activation of genes in proinflammatory pathways (Orr et al., 2002). Intranigral injection of LPS causes strong microglial activation and subsequent dopaminergic neurodegeneration in both SN and striatum (Herrera et al., 2000). Intraperitoneal injection of LPS has been reported to produce an increase in gene expression for Toll-like receptor 2, IFN-γ, COX-2, and IL-6 receptor in the CNS in rodents (Vallieres and Rivest, 1997). LPS also induces expression of caspase-11 and increased IL-1β level (Arai et al., 2004). Similarly, in our experiment, LPS induced nitrile level and iNOS expression along with increased mRNA expression of different proinflammatory cytokines in microglial cells.
MPTP is a lipophilic molecule which crosses the blood–brain barrier (BBB) and reaches the brain within minutes. Once in the brain, MPTP is converted to MPP\(^+\) by monoamine oxidase B (MAO-B) in astroglial cells (Markey et al., 1984). The latter is believed to be responsible for glial activation and neuronal death (Dauer and Przedborski, 2003). In our experiment, we also found activated microglial and astroglial cells expressing high levels of Pin1 protein following MPTP administration. Previously, marked activation of nigral NF-\(\kappa\)B has been reported in postmortem PD brains and in MPTP mouse model of PD (Ghosh et al., 2007). We also showed that parkinsonian neurotoxin MPP\(^+\) was capable of activating NF-\(\kappa\)B p65 in BV2 microglial cells and post natal mouse microglial cells.

However, Juglone, a pharmacological inhibitor of Pin1 was capable of attenuating MPP\(^+\) induced NF-\(\kappa\)B p65 expression in microglial cells. Once NF-\(\kappa\)B is activated, in collaboration with other proinflammatory transcription factors, NF-\(\kappa\)B drives the transcription of several proinflammatory molecules including iNOS, TNF-\(\alpha\), and IL-1\(\beta\) in both microglia and astroglia (Liu et al., 2006). As glial cells mediated production of proinflammatory molecules and further activation of gliosis are important features of PD pathology, it appears that NF-\(\kappa\)B activation regulates these pathological features either directly or indirectly and that Pin1 inhibition (by means of pharmacological inhibitor- Juglone) rescues against inflammatory reactions via suppression of NF-\(\kappa\)B dependent pathological steps.

In our study, we used Juglone (5-hydroxy-1, 4-napthalenedione, \(\text{C}_{10}\text{H}_{8}\text{O}_{3}\)), a pharmacological inhibitor of Pin1 and it is an aromatic organic naphthoquinone, naturally found in the leaves, roots and bark of plants in the black walnut and has been widely used as a Pin1 inhibitor (Esnault et al., 2007b; Jeong et al., 2009). The enzymatic activity of E. coli parvulin and human Pin1 is specifically inactivated by Juglone by modification of thiol
groups of cysteine residue in parvulin (Hennig et al., 1998). Recent report demonstrated that Juglone prevented acute and chronic rejection of MHC mismatched, orthotopic rat lung transplants by reducing the expression of IFN-γ and CXCL-10 mRNA stability, accumulation and protein expression after cell activation (Esnault et al., 2007a). Juglone also attenuates rheumatoid arthritis development in human primary culture RA chondrocytes and CII treated DBA/1J mice (Jeong et al., 2009). Additionally, recent reports suggested anti-inflammatory, anti-viral and anti-fungal properties of Juglone (Omar et al., 2000). In our study, we found 3µM Juglone is non-toxic to the microglial cells. Additionally, we found that Juglone attenuated LPS or TNF-α or MPP⁺ induced Pin1 expression in BV2 and primary glial cells. Juglone also attenuated LPS induced nitrite and iNOS expression in microglial cells. Recent report demonstrated that 5mg/kg dose of Juglone blocked type-2 rheumatoid arthritis in DBA/1J mice (Jeong et al., 2009). In our study, we injected multiple doses of Juglone (3mg/kg) intraperitoneally to the C57BL/6 mice. Importantly, these doses of Juglone were nontoxic. Here we demonstrate that Juglone effectively attenuates the MPTP-induced mRNA expression of proinflammatory molecules and cytokines in nigrostriatum. Furthermore, Juglone also attenuated MPTP induced iNOS expression in microglial and astroglial cells.

In conclusion, we have demonstrated for the first time in our knowledge that Pin1 and p65 are upregulated in BV2 and mouse primary glial cells in response to LPS or TNF-α or MPP⁺ stimuli. We showed specific interaction between p65 and Pin1 in BV2 microglial cells. By employing pharmacological inhibitor of Pin1, we were able to reduce the expression of Pin1 and p65. Juglone also attenuated LPS induced nitrite production and subsequent iNOS
expression in microglial cells. LPS induced expression of proinflammatory cytokine was also attenuated by Juglone, suggesting Pin1’s possible role in modulating inflammation. Additionally, we also demonstrated increased expression levels of Pin1 in nigral microglial and astroglial cells of MPTP treated mice. Juglone was also capable of attenuating MPTP induced expression of proinflammatory cytokines in mice midbrain. Additionally, Juglone attenuated MPTP-induced nigral iNOS expression in vivo. These results clearly suggest that Pin1 may serve as a novel pharmacological target for attenuating neuroinflammation in PD and Pin1 inhibitor Juglone may be used as novel neuroprotective agent.

References.


Figure 1. Induction of Pin1 expression in response to different stimuli in BV2 cells.

A. BV2 cells were treated with 1 µg/ml LPS for 24 h. After treatment, cells were collected and expression of Pin1 was checked by western blotting. B. Bar graph showing means
Western blot Pin1/β-actin ratios ± SEM from 2 different experiments. C. BV2 cells were treated with 10µM MPP+ for 24 h. After treatment, cells were collected and expression of Pin1 was checked by western blotting from cell lysates. D. Bar graph showing means Western blot Pin1/β-actin ratios ± SEM from 2 different experiments. E. BV2 cells were treated with 3 different doses (10ng/ml, 20ng/ml and 30ng/ml) of TNF-α for 18 h. After treatment, cell lysates were made and expression of Pin1 was checked by western blotting. F. Bar graph showing means Western blot Pin1/β-actin ratios ± SEM from 3 different experiments. a, p < 0.01 vs control; c, p < 0.01 vs TNF-α (different doses).
Figure 2. Parkinsonian toxicant MPP+ induces Pin1 expression in primary mesencephalic mixed neuro-glial cultures. Mesencephalic tissues from E15 mouse embryos were dissected out and grown on laminin-coated coverslips. The mixed neuro-glial cultures were treated with 10μM MPP+ for 24 h. After treatment, cells were fixed with 4% paraformaldehyde. A. Double labeling of Iba-1 and Pin1 in primary mesencephalic mixed neuro-glial culture. B. Double labeling of GFAP and Pin1 in primary mesencephalic mixed neuro-glial culture. C. Expression of Pin1 in primary astrocytes culture from C57BL/6 postnatal day 1 (P1) mouse pups.
Figure 3

**A.** BV2 cells were treated with 10μM MPP+ for 24h. After treatment cells were collected and expression of Pin1 was checked by western blotting. 

**B.** BV2 cells were treated with 1 µg/ml LPS for 24 h. After treatment, cells were collected and expression of Pin1 was checked by western blotting. 

**C.** BV2 cells were treated with 3 different doses (10ng/ml, 20ng/ml and 30ng/ml) of TNF-α for 18 h. After treatment, cell lysates were made and expression of Pin1 was checked by western blotting. 

**D.** BV2 cells were treated with 10ng/ml dose of TNF-α for different time points (6h, 12h, 18h and 24h).
Figure 4

A

**IP: p65**

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B

**IP: Pin1**

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**Figure 4. Pin1 associates with p65 in BV2 cells.** A. BV2 cells were grown in T25 flasks. When cell density reached approximately 80%, the cells were treated with either 10μM MPP+ for 24h or 1μg/ml LPS for 24h or 10ng/ml TNF-α for 18h. After treatment, cells were lysed in modified RIPA buffer and lysates from each sample was then subjected to immunoprecipitation (IP) using antibodies specific for Pin1. IP samples were probed with antibodies to p65 to demonstrate a specific interaction between Pin1 and p65. B. BV2 cells were grown in T25 flasks. When cell density reached approximately 80%, the cells were treated with either 10μM MPP+ for 24h or 1μg/ml LPS for 24h or 10ng/ml TNF-α for 18h. After treatment, cells were lysed in modified RIPA buffer and lysates from each sample was then subjected to immunoprecipitation (IP) using antibodies specific for p65. IP samples were probed with antibodies to Pin1. Results are representative of two separate experiments.
Figure 5

A

Bar chart showing the number of Sytox positive cells in each well for different conditions.

B

Images showing fluorescence and phase contrast images under different conditions.

C

Table showing the effects of MPP+ and Juglone on Pin1 and β-actin expression.

D

Western blot images showing the expression levels of Pin1 and β-actin under Con, LPS, and LPS + Jug conditions.

E

Western blot images showing the expression levels of Pin1 and β-actin under Con, TNF-α, and TNF-α + Jug conditions.

F

Immunofluorescence images showing the expression of IBA-1, GFAP, and Pin1 under Control, MPP+, and MPP+ + Jug conditions.
Figure 5. Pin1 inhibitor Juglone attenuates MPP+ or LPS or TNF-α induced Pin1 expression and cytotoxicity in BV2 and primary microglial cells. BV2 cells were treated with 10ng/ml TNF-α for 18 hrs in the presence or absence of different doses (1µM, 3µM, 10µM and 20µM) of Juglone. A. Cytotoxicity of BV2 cells during 10µM MPP+ treatment in the presence or absence of above mentioned four different doses of Juglone. Cytotoxicity was determined by Sytox green nucleic acid stain by measuring the fluorescence at regular intervals, as described in Materials and Methods. B. The two panels demonstrate phase contrast images (right) and fluorescence micrographs (left) to show the extent of Sytox green staining of cells in the field of view. C. BV2 cells were treated with10µM MPP+ for 24h in the presence or absence of different doses (1µM, 3µM and 10µM) of Juglone. After treatment, cell lysates were prepared and immunoblotted for Pin1. D. BV2 cells were treated with 1µg/ml LPS for 24h in the presence or absence of 3µM Juglone. After treatment, cell lysates were prepared and immunoblotted for Pin1. E. BV2 cells were treated with 10ng/ml TNF-α for 18h in the presence or absence of 3µM Juglone. After treatment, cell lysates were prepared and immunoblotted for Pin1. F. Primary glial cells from C57BL/6 postnatal day 1 (P1) mouse pups were immunolabelled for IBA-1(marker of microglia) and Pin1 (upper panel) and for GFAP (marker of astrocytes) and Pin1. Data represent the mean ± SEM of three different experiments. **, p < 0.01 vs. TNF-α; ***, p < 0.001 vs. TNF-α.
Figure 6. Juglone inhibits TNF-α or LPS or MPP+ induced activation of p65 in BV2 cells. A. BV2 cells were treated with 10ng/ml TNF-α in presence or absence of 3µM Juglone. 18h after treatment, cells were immunostained for Pin1 and p65. B. BV2 cells were treated with 10µM MPP+ for 24h in the presence or absence of different doses (1µM, 3µM and 10µM) of Juglone. After treatment, cell lysates were prepared and immunoblotted for p65. C. Bar graph showing means Western blot p65/β-actin ratios ± SEM from 3 different experiments. D. BV2 cells were treated with 1µg/ml LPS for 24h in the presence or absence of 3µM Juglone. After treatment, cell lysates were prepared and immunoblotted for p65. E. Bar graph showing means Western blot p65/β-actin ratios ± SEM from 3 different experiments. F. BV2 cells were treated with 10ng/ml TNF-α for 18h in the presence or absence of 3µM Juglone. After treatment, cell lysates were prepared and immunoblotted for p65. G. Bar graph showing means Western blot Pin1/β-actin ratios ± SEM from 3 different experiments. ***, p < 0.001 vs. control; **, p < 0.01 vs. control; @, p < 0.05 vs. MPP+ or TNF-α; #, p < 0.01 vs. MPP+; $, p < 0.05 vs LPS.
Figure 7. Juglone attenuates generation of nitric oxide in BV2 and primary microglial culture. A. BV2 cells were treated with LPS (1µg/ml) in presence or absence of 3 different doses (1µM, 3µM and 10µM) of Juglone. After 24 h of treatment nitric oxide production was
determined indirectly by quantifying the nitrite levels in the supernatant using the Griess reagent and a sodium nitrite standard curve. **B.** BV2 cells were treated with TNF-α (10ng/ml) in presence or absence of 4 different doses (1μM, 3μM, 5μM and 10μM) of Juglone. After 18 h of treatment nitric oxide production was determined indirectly by quantifying the nitrite levels in the supernatant using the Griess reagent and a sodium nitrite standard curve. **C.** Primary microglial cells from C57BL/6 one day old pups were treated with LPS (100ng/ml) in presence or absence of 3μM of Juglone. After 12 h of treatment nitric oxide production was determined indirectly by quantifying the nitrite levels in the supernatant using the Griess reagent and a sodium nitrite standard curve. **D.** BV2 cells were treated with 10μM MPP+ for 24h in the presence or absence of different doses 3μM of Juglone. After treatment, cell lysates were prepared and immunoblotted for iNOS. **E.** Bar graph showing means Western blot iNOS/β-actin ratios ± SEM from 3 different experiments. **F.** BV2 cells were treated with 1μg/ml LPS for 24h in the presence or absence of 3μM Juglone. After treatment, cell lysates were prepared and immunoblotted for iNOS. **G.** Bar graph showing means Western blot iNOS/β-actin ratios ± SEM from 3 different experiments. **H.** BV2 cells were treated with 10ng/ml TNF-α in presence or absence of 3μM Juglone. 18h after treatment, cells were immunoblotted for iNOS. **I.** Bar graph showing means Western blot iNOS/β-actin ratios ± SEM from 3 different experiments. ***,** p < 0.001 vs control; **,** p < 0.01 vs control; *, p < 0.05 vs TNF-α or LPS; #, p < 0.01 vs LPS; @, p < 0.001 vs TNF-α
Figure 8. Juglone inhibits LPS induced cytokine production in BV2 cells and primary microglia culture. BV2 cells were seeded in equal numbers on poly-d-lysine coated plates. The cells were treated with LPS (300ng/ml) for 24 h in the presence or absence of 3μM Juglone. After treatment, cytokine levels in the supernatant were quantified using the luminex immunoassay system. Levels of IL-6 (A), TNF-α (B) and IL-12 (C) in the supernatant. Primary microglia culture was initiated from C57BL/6 one day old pup and seeded in equal numbers on poly-d-lysine coated plates. The cells were then treated with LPS (100ng/ml) for 24 h in the presence or absence of 3μM Juglone. After treatment, cytokine levels in the supernatant were quantified using the luminex immunoassay system. Levels of IL-6 (D), TNF-α (E) and IL-12 (F) in the supernatant. Data represent the mean ± SEM of three different experiments. ***, p < 0.001 vs. control; **, p < 0.01 vs. control; *, p < 0.05 vs. LPS.
Figure 9. Increased expression of Pin1 in glial cells of MPTP-treated mice. C57BL/6 mice were treated with 4 doses of MPTP (18mg/kg/dose) at 2 h interval via i.p. and 24 h after MPTP treatment mice were sacrificed. **A.** Double labeling of GFAP and Pin1 in substantia nigra (SN) region of brain. A single enlarged GFAP positive asatroglial cells expresses Pin1 in the cytosol (inset). **B.** Double labeling of IBA-1 and Pin1 in SN region of brain. A single enlarged IBA-1 positive microglial cell expresses Pin1 in the cytosol (inset).
Figure 10. Activation of p65 in nigrostriatum of MPTP-treated mice. Mice were treated with 4 doses of MPTP (18mg/kg/dose) at 2 h interval via i.p. and 24 h after MPTP treatment mice were sacrificed. A. Western blotting of p65 in substantia nigra. B. Western blotting of p65 in striatum. Mice receiving PBS as a control, MPTP (18mg/kg/dose) or MPTP and Juglone (3mg/kg/day) were sacrificed 24 h after the last dose of MPTP administration and ventral midbrain region was dissected out. C. The mRNA expression of TNF-α, IL-1β, IL-6 and iNOS was analyzed by quantitative real-time PCR. Data represent the mean ± SEM of six mice per group. **, p < 0.01 vs. control; ***, p < 0.001 vs. control; @, p < 0.05 vs MPTP; a < 0.01 vs MPTP.
Figure 11. Juglone attenuates MPTP-induced iNOS expression in glial cells. Mice were treated with MPTP (18mg/kg/dose) at 2 h interval via i.p. in the presence or absence of Juglone (3mg/kg/day). 24 h after the last dose of MPTP injection mice were sacrificed and substantia nigra (SN) tissue sections were processed for immunostaining. A. Double labeling of GFAP and iNOS in SN. B. Double labeling of IBA-1 and iNOS in SN. Inset pictures demonstrated colocalization of GFAP/IBA-1 and iNOS. Images were captured at 20X and 60X (insets) magnifications.
CHAPTER IV

ANTI-INFLAMMATORY AND NEUROPROTECTIVE EFFECTS OF A NOVEL COMPOUND DIAPOCYNIN IN A MOUSE MODEL OF PARKINSON’S DISEASE

A manuscript communicated to The Proceedings of Natl. Academy of Science

Anamitra Ghosh\textsuperscript{1}, Arthi Kanthasamy\textsuperscript{1}, Pallavi Srivastava\textsuperscript{1}, Joy Joseph\textsuperscript{2}, Vellareddy Anantharam\textsuperscript{1}, Balaraman Kalyanaraman\textsuperscript{2} and Anumantha G. Kanthasamy\textsuperscript{1}

\textsuperscript{1}Department of Biomedical Sciences, Iowa Center for Advanced Neurotoxicology, Iowa State University, Ames, IA., \textsuperscript{2}Department of Biophysics, Medical College of Wisconsin, Milwaukee, WI

Abstract

Parkinson’s disease (PD) is a devastating neurodegenerative disorder marked by progressive increases in motor deficits affecting several million people worldwide. Recent studies suggest that glial cell activation and its inflammatory response may contribute to the progressive degeneration of dopaminergic neurons in PD. Currently, there are no neuroprotective agents available that can slow down the disease progression. In the present study, we evaluated the antioxidant and anti-inflammatory efficacy of diapocynin, an oxidative metabolite of a naturally occurring agent apocynin, in a preclinical 1-methyl-4phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD. First, oral administration of diapocynin (300 mg/kg/day) significantly attenuated MPTP-induced microglial and astroglial
cell activation in substantia nigra. Second, MPTP induced expression of gp91phox and iNOS activation in the substantia nigra were also attenuated by diapocynin. Diapocynin markedly inhibited MPTP-induced 3-nitrotyrosine production and 4-hydroxynonenol (diagnostic markers of oxidative and nitrative damage) in the substantia nigra. Treatment with diapocynin also significantly improved the locomotor activity, restored dopamine and its metabolites and protected the dopaminergic neurons in nigrostriatum in this preclinical model of PD. Finally, diapocynin administered 3 days after initiation of disease was able to restore the neurotransmitter levels. Collectively, these results demonstrate that diapocynin exhibits profound anti-inflammatory, antioxidant and neuroprotective effects in a well-established animal model of PD. These findings have immediate translational relevance for treating PD patients.

**Introduction**

Parkinson’s disease (PD) is the most common neurodegenerative movement disorder estimated to affect 1% of population over 65 years of age. It is chronic and progressive in nature and is characterized predominantly by resting tremors, bradykinesia, muscular rigidity and postural instability along with several non-motor symptoms (Vila and Przedborski, 2004). The pathological hallmarks of PD are the depletion of striatal dopamine caused by degeneration of dopaminergic neurons in the substantia nigra (SN) region of midbrain, appearance of cytoplasmic inclusions known as Lewy bodies in surviving neurons of the SN, and activation of glial cells (Dauer and Przedborski, 2003; Ghosh et al., 2009).

Although the etiologic mechanisms of PD are poorly understood, recent reports implicate brain inflammation and oxidative stress in disease pathogenesis (Dauer and Przedborski, 2003; Gao et al., 2003a). Microglia and astrocytes are major mediators of
neuroinflammation in PD. Several reports demonstrated the activation of microglial cells and astroglial cells in close proximity to the damaged or dying dopaminergic neurons in substantia nigra (McGeer et al., 1988). The levels of nitrite (NO$_2$), a metabolite of NO, and inducible nitric oxide synthase (iNOS) are higher in the central nervous system (CNS) of human PD cases and in animal models of PD (Hunot et al., 1996). Consistent with this finding, iNOS knockout animals were resistant to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neuronal loss in the substantia nigra (Dehmer et al., 2000). One of the major sources of reactive oxygen species (ROS) in neurodegeneration is NADPH oxidase, a multimeric enzyme that generates both O$_2^-$ and H$_2$O$_2$ (Bokoch and Knaus, 2003). Reaction between superoxide (O$_2^-$) and NO forms a more potent species peroxynitrite (ONOO$^-$), another key player in dopaminergic neurodegeneration in PD. Moreover, 4-hydroxynonenol (4-HNE), an unsaturated aldehyde derived from lipid hydroperoxidase is reported to mediate the induction of neuronal apoptosis in the presence of oxidative stress (Awasthi et al., 2008). Proinflammatory cytokines (TNF-α, IL-1β, IFN-γ, IL-6), chemokines and prostaglandins levels are also elevated in PD brains (Nagatsu et al., 2000). Recent reports suggest that nuclear factor-κB (NF-κB), a transcription factor that is required for transcription of proinflammatory molecules is also activated in the substantia nigra of PD patients and MPTP-treated mice (Ghosh et al., 2007). Additionally, Nurr1, a nuclear orphan receptor suppresses glial activation by docking to NF-κB-p65 on target inflammatory gene promoters and protects against dopaminergic neurodegeneration in substantia nigra (Saijo et al., 2009). These findings strongly suggest that mitigation of neuroinflammation and oxidative stress may be a viable neuroprotective strategy for treatment of PD.
Several inhibitors of NADPH oxidase have previously been tested for their anti-inflammatory and antioxidant effects in dopaminergic cells (Anantharam et al., 2007). For example, apocynin (4-hydroxy-3-methoxyacetophenone), a plant derived antioxidant, has been widely used as an NADPH oxidase inhibitor in *in vitro* and *in vivo* experimental models of PD (Lafeber et al., 1999; Zhang et al., 2005; Anantharam et al., 2007). High concentrations of apocynin have been shown to protect against neuronal damage in SOD1 mutant ALS mice model (Lin et al., 2006). However, apocynin failed to protect the dopaminergic neurons against rotenone-mediated neurotoxicity in the absence of glial cells (Gao et al., 2003a). Other studies have shown mixed results with regard to apocynin (Vejrazka et al., 2005). *In vivo*, apocynin has been shown to form diapocynin, a dimer of apocynin, resulting in the inhibition of NADPH oxidase. In the present study, we used diapocynin, a dimeric derivative of apocynin and investigated its antioxidant and anti-inflammatory roles in mouse model of PD. Results show that diapocynin suppresses MPTP induced glial activation, attenuates nigral expression of proinflammatory molecules, reduces oxidative stress and exhibits significant protection of the nigrostriatal axis after MPTP administration. Additionally, diapocynin also slows down the progression of the disease in MPTP model. Collectively, these results suggest that additional preclinical development of diapocynin may yield an effective neuroprotective and anti-neuroinflammatory drug capable of arresting the progression of PD.

**Materials and methods:**

**Animals and treatment.** Eight-week-old male C57BL/6 mice weighing 24 to 28 g were housed in standard conditions: constant temperature (22 ± 1°C), humidity (relative, 30%),
and a 12-h light/dark cycle. Use of the animals and protocol procedures were approved and supervised by the Committee on Animal Care at Iowa State University (Ames, IA). Mice received diapocynin (300mg/kg/day) in 10% ethanol by oral gavage either 1 day before or after 3 days of the MPTP insult. For MPTP treatment, mice received 25mg/kg/day MPTP-HCl in saline intraperitoneally for consecutive 5 days. Control mice received equvolume injection of saline.

**HPLC analysis of striatal dopamine and its metabolites levels.** Samples were prepared and quantified as described previously (Ghosh et al., 2010; Zhang et al., 2007). In brief, 7 days after MPTP injection mice were sacrificed and striata were collected and stored at -80C. On the day of analysis, neurotransmitters from striatal tissues were extracted using an antioxidant extraction solution (0.1 M perchloric acid containing 0.05% Na₂EDTA and 0.1% Na₂S₂O₅) and isoproterenol (as internal standard). DA, DOPAC and HVA were separated isocratically by a reversed-phase column with a flow rate of 0.6 ml/min. An HPLC system (ESA Inc., Bedford, MA) with an automatic sampler equipped with refrigerated temperature control (model 542; ESA Inc.) was used for these experiments. The electrochemical detection system consisted of a Coulochem model 5100A with a microanalysis cell (model 5014A) and a guard cell (model 5020) (ESA Inc.). The data acquisition and analysis were performed using the EZStart HPLC Software (ESA Inc.).

**HPLC/MS analysis of Diapocynin.** Amount of diapocynin from Striatum and substantia nigra was quantified using the Agilent 1100 series LCMS binary pump, PDA detector (UV diode array detector) and an auto sampler. On the day of analysis, 20µL sample was passed through the 0.2µm filter at the eluent flow rate of 0.25 ml/min. Negative-ion, atmospheric
pressure chemical ionization was used at amplitude 1.5Volts and Manual MS/MS was done. The mobile phase used in LC-MS consisted of a gradient elution. Solvent A was 480: 20: 0.38 water: methanol: ammonium acetate (v/ v/ w) and solvent B was 20: 480: 0.38 water: methanol: ammonium acetate (v/ v/ w). The standards series were taken as 0.3µg, 1.0µg, 3.0µg, 10 µg, 30 µg. The actual molecular wt of diapocynin is 329.1 but by breaking the molecule in MS/MS it becomes 313.9 g/mol by elimination of one methyl molecule. The retention time for diapocynin peak was 1.9 min. Data were fit to a straight line by linear regression analysis using Quant analysis software.

**Western blotting.** Mice were sacrificed 4 days or 7 days after MPTP treatment and substantia nigra tissue was dissected out. Brain lysates containing equal amounts of protein were loaded in each lane and separated in a 10 to 15% SDS-polyacrylamide gel electrophoresis gel as described previously (Jin et al., 2011 ; Zhang et al., 2007). Proteins were transferred to a nitrocellulose membrane and nonspecific binding sites were blocked by treating with Licor odyssey blocking buffer. The membranes were then incubated with different primary antibodies such as anti-TH (Chemicon), anti-IBA-1 (Abcam), anti-GFAP (Chemicon), anti-iNOS (Santa Cruz), anti-gp91phox (Abcam), anti-3NT (Chemicon) and anti-4HNE (R&D). Next, membranes were incubated with Alexa Fluor 680 goat anti-mouse or Alexa Fluor 680 donkey anti-goat (Invitrogen) or IR dye 800 donkey anti-rabbit (Rockland) secondary antibodies. To confirm equal protein loading, blots were reprobed with a β-actin antibody (Sigma; 1:10000 dilution). Western blot images were captured with a Licor Odyssey machine (Licor, NE). The western blot bands were quantified using NIH Image J software.
**Immunohistochemistry.** One day after last MPTP treatment, mice were perfused with 4% paraformaldehyde and post fixed with PFA and 30% sucrose respectively. Next, fixed brains were cut into 30µ sections and were incubated with different primary antibodies such as anti-IBA-1 antibody (Abcam), anti-GFAP (Chemicon), anti-iNOS (Santa Cruz), anti-3NT (Chemicon), anti-gp91phox (Abcam) and anti-4HNE (R & D) for overnight at 4C. Appropriate secondary antibodies (Alexa Fluor 488 or 594 or 555 from Invitrogen) were used followed by incubation with 10µg/ml Hoechst 33342 for 5 min at room temperature to stain the nucleus. Sections were viewed under a Nikon inverted fluorescence microscope (model TE-2000U; Nikon, Tokyo, Japan); images were captured with a SPOT digital camera (Diagnostic Instruments, Inc., Sterlings Heights, MI).

**Fluoro-Jade B and TH double labeling.** On the day of staining, sections were incubated with anti-TH antibody (Chemicon) followed by Alexa Fluor 568 donkey anti-mouse (Invitrogen) secondary antibody. Then Fluoro-Jade B staining was done on the same sections by modified FJB stain protocol including incubation in 0.06% potassium permanganate for 2 min and 0.0002% FJB stain for 5 min. Sections were viewed under a Nikon inverted fluorescence microscope (model TE-2000U; Nikon, Tokyo, Japan); images were captured with a SPOT digital camera (Diagnostic Instruments, Inc., Sterlings Heights, MI).

**DAB immunostaining and stereological counting.** DAB immunostaining was performed in striatal and substantia nigral sections as described previously (Ghosh et al., ; Ghosh et al., 2009). In brief, 30 µm sections were incubated with either anti-TH antibody (Calbiochem, rabbit anti-mouse, 1:1800) or anti-IBA-1 (Abcam, goat anti-mouse, 1:1000) or anti-GFAP...
(Chemicon, mouse anti-mouse, 1:1000) antibody for overnight at 4C. Next, sections were incubated in biotinylated anti-rabbit or goat or mouse secondary antibody followed by incubation with avidin peroxidase (Vectastatin ABC Elite kit, Vector laboratories). Immunolabeling was observed using diaminobenzidine (DAB), which yielded a brown stain. Total numbers of TH positive neurons in SN were counted stereologically with Stereo Investigator software (MBF Bioscience, Williston, VT, USA), using an optical fractionators (Ghosh et al., 2010; Ghosh et al., 2007).

Behavioral measurements. An automated device (AccuScan, model RXYZCM-16, Columbus, OH) was used to measure the spontaneous activity of mice. The activity chamber was 40×40×30.5 cm, made of clear Plexiglas and covered with a Plexiglas lid with holes for ventilation. Data were collected and analyzed by a VersaMax Analyzer (AccuScan, model CDA-8, Columbus, OH). Before any treatment, mice were placed inside the infrared monitor for 10 min daily and 5 min daily for 3 consecutive days to train them. After 5 days of last MPTP injection, openfield and rotarod experiments were conducted. Locomotor activities were presented for 10 min test secession. For rotarod experiment 20 rpm speed was used. Mice were given a 5-7 min rest interval to eliminate stress and fatigue.

Data analysis. Data analysis was performed using Prism 4.0 software (GraphPad Software Inc., San Diego, CA). Raw data were first analyzed using one-way analysis of variance and then Tukey’s post-test was performed to compare all treatment groups. Differences with $p < 0.05$ were considered significant.

Results
Diapocynin inhibits the glial activation in the substantia nigra of MPTP-induced mouse model of PD.

The treatment schedule of mice with diapocynin is shown in Fig. 1A. We first determined whether diapocynin crosses the blood brain barrier. The LC-MS results show substantial accumulation of diapocynin in substantia nigra (SN) and striatum (ST) of brain after 13 days of oral gavage treatment of diapocynin (300mg/kg/day) (SI Fig. 7). Then we determined if diapocynin protected against MPTP-induced microglial activation and astrogliosis. MPTP treatment induced increased expression of IBA-1 positive microglial cells (Fig. 1B-D) and GFAP positive astrocytes (Fig. 1E-G) in SN of MPTP treated mice. In addition to that, DAB immunostaining of IBA-1 showed increased number of amoeboid shaped microglial cells with thick processes, characterization of microgliosis, and DAB immunostaining of GFAP showed bigger cell bodies of astroglial cells with increased and thick processes, characterization of astrogliosis (Fig. 1B and E). Next, we investigated whether diapocynin could attenuate MPTP-induced glial activation in mice SN. As shown by DAB immunostaining experiment, diapocynin strongly inhibited MPTP induced microgliosis, as the IBA-1 positive microglial cells were very few in number, with smaller cell bodies and thin processes (Fig. 1B). Diapocynin significantly decreased MPTP-induced increases in GFAP positive astroglial cells in the mouse SN (Fig. 1E). Western blot analysis for IBA-1 and GFAP also shows that diapocynin suppressed nigral IBA-1 (Fig. 1C and D) and GFAP (Fig. 1F and G) protein expression in MPTP-treated mice. These results suggest that diapocynin significantly attenuates MPTP-induced increases in glial cell population in the mouse SN.
Diapocynin inhibits MPTP-induced increases in iNOS expression in mouse SN.

Previous reports have demonstrated the role of inflammation in the dopaminergic neurodegeneration in PD and its animal models (Hunot et al., 1996; Ghosh et al., 2007). Inducible nitric oxide synthase (iNOS), an important proinflammatory molecule, is typically elevated in disease conditions (Ghosh et al., 2009). We observed a marked increase in the expression of iNOS in SN of MPTP-treated mice versus saline-treated control mice (Fig. 2A and B). However, diapocynin attenuated MPTP induced expression of iNOS protein (Fig. 2A and B). Additionally, immunofluorescence analysis for iNOS in the SN sections shows that MPTP treatment led to marked increase in nigral iNOS protein expression and that iNOS colocalized with GFAP positive astrocytes and IBA-1 positive microglial cells (Fig. 2C and D). However, consistent with their inhibitory effect on the activation of glial cells, diapocynin suppressed MPTP induced expression of iNOS (Fig. 2C and D). These results demonstrate that diapocynin effectively suppresses the expression of proinflammatory molecule iNOS in vivo in the SN of MPTP treated mice.

Diapocynin attenuates MPTP-induced activation of microglial NADPH oxidase.

NADPH oxidase is the major source of reactive oxygen species (ROS) and recent findings suggested that NADPH oxidase-induced oxidative stress played a pivotal role in nigral dopaminergic neurodegeneration in PD and its animal models (Wu et al., 2003). The western blot analysis showed an increased expression of gp91phox, a membrane bound subunit of NADPH oxidase after MPTP injection (Fig 3A-B) compared to weak expression in saline treated mice (Fig. 3A-C). Robust gp91phox immunoreactivity was seen specifically in larger cells with thick, shorter ramifications in the SN of MPTP treated mice (Fig. 3C).
Double immunolabeling studies confirmed that gp91phox immunoreactivity appeared to colocalize with IBA-1 positive microglia (Fig. 3C, middle panel). In MPTP model of PD, gp91phox does not colocalize either with astrocytes or dopaminergic neurons (Wu et al., 2003). Diapocynin treatment attenuated MPTP induced gp91phox protein expression in the IBA-1 positive microglial cells in the SN (Fig. 3A-C). These results suggest that diapocynin effectively blocks the MPTP-induced microglial NADPH oxidase.

**Diapocynin inhibits formation of peroxynitrite and 4-hydroxynonenol (4-HNE) in the nigral dopaminergic neurons of MPTP treated mice.**

3-NT has been widely used as a marker of nitric oxide-dependent oxidative stress (Ara et al., 1998). The western blot analysis demonstrates increased expression of 3-NT protein in the SN of MPTP treated mice that were markedly decreased following diapocynin treatment (Fig 4A and B). Further confirmation came from immunolabeling of 3-NT in the SN sections. In the MPTP treated sections, we observed increased expression of 3-NT particularly in the SN region of ventral midbrain and that 3-NT colocalized (yellow color) with tyrosine hydroxylase (TH) positive dopaminergic neurons (Fig. 4C). However, 3-NT expression was observed in very few TH positive dopaminergic neurons in the MPTP-treated mice that also received diapocynin (Fig. 4C). These results strongly suggest that diapocynin inhibits peroxynitrite (ONOO-) formation induced by MPTP in dopaminergic neurons in the SN. Along with peroxynitrite, 4-HNE formation was also increased (Fig. 4D and E). MPTP treatment led to marked increase in protein expression of 4-HNE in the SN. However, diapocynin strongly inhibited MPTP induced expression of this unsaturated aldehyde in the SN (Fig. 4D and E). Consistent with these findings, immunofluorescence
analysis of the SN sections (Fig. 4F) showed that level of expression of 4-HNE was much higher in the MPTP treated SN sections than saline treated control sections and that 4-HNE colocalized with TH-positive dopaminergic neurons. However, treatment of MPTP treated mice with diapocynin led to the attenuation of 4-HNE in the dopaminergic neurons (Fig. 4F). These results suggest that diapocynin mitigates oxidative stress in nigral dopaminergic neurons of MPTP-intoxicated mice.

**Diapocynin improves locomotor activities in MPTP-injected mice.**

To assess the effectiveness of diapocynin against motor deficits induced by MPTP, we measured the locomotor and open field activities. We used VersaMax infrared computerized activity monitoring system (Accuscan, OH) and a rotarod instrument (Accuscan, OH) for measurement of locomotor activities and motor coordination, respectively. Animals were subjected to behavioral measurements 4 days after the last does of MPTP treatment (Fig. 1A). Representative motor activity maps of movement of saline treated control, MPTP and MPTP plus diapocynin treated mice are shown in Fig 5A. As observed in the VersaPlot (Fig 5A), we found decreased movement of MPTP treated mice and improvement of locomotion in MPTP plus diapocynin treated group. Subacute MPTP treatment resulted in marked decrease in horizontal activity (Fig. 5B), vertical activity (Fig. 5C), total distance travelled (Fig. 5D), movement time (Fig. 5E), number of stereotypy (Fig. 5G), number of rearing (Fig. 5H) and rotarod performances at 20 rpm speed (Fig. 5I) consistent with our previous observations (20). Additionally, rest time was increased in the MPTP treated mice (Fig. 5F). Again, diapocynin significantly restored MPTP induced locomotor and motor co-ordination impairments (Fig 5B-I). In addition to that, we found
significant restoration of behavioral imparities with diapocynin treatment in a transgenic LRRK2 mice model of PD (Data not shown).

**Diapocynin protects against MPTP-induced neurodegeneration.**

Seven days after MPTP treatment, mice were sacrificed and brains were processed for tyrosine hydroxylase (TH) immunostaining, western blotting and HPLC neurochemical analysis (Fig. 6A-H). As observed earlier (Ghosh et al., 2010), MPTP treatment led to degeneration of TH positive dopaminergic neurons and its terminals in the SN and the striatum (ST) (Fig. 6A, 2X magnification). Higher magnified 10X pictures (Fig. SI 8A) clearly demonstrated loss of neurons in substantia nigra pars compacta (SNpc), substantia nigra lateralis (SNl) and substantia nigra reticularis (SNr) regions of nigral tract. Additionally, stereological counting of TH positive neurons in SN of MPTP treated mice also showed > 60% reduction (Fig. 6E). However, in MPTP treated mice that also received diapocynin, nigral TH positive neurons and striatal TH terminals were restored to near control levels (Fig. 6A & E and Fig. SI 8A). Consistent with these findings, western blot of TH in nigra and striatum also showed significant decrease of TH protein levels in MPTP treated mice (Fig. 6B-D). However, orally administered diapocynin significantly restored the nigral and striatal TH protein levels in MPTP treated mice (Fig. 6B-D). To further confirm that diapocynin protects against MPTP-induced dopaminergic neurodegeneration, we performed Fluoro-Jade B (FJB) staining in nigral dopaminergic neurons. FJB effectively stains the degenerating neurons and it is also used as a marker of neuronal damage (Kanthasamy et al., 1999). As anticipated, we detected a decreased number of TH positive neurons in the MPTP treated sections. However, FJB positive cells were increased in the
MPTP treated sections, suggesting neurodegeneration in the SN (Fig. SI 8B). Additionally, we also observed some TH positive cells that showed FJB staining, indicating dying dopaminergic neurons. However, MPTP-treated mice that also received diapocynin demonstrated fewer FJB positive cells, suggesting survival of neurons.

Next we determined whether diapocynin protects against biochemical deficits caused by MPTP, we quantified levels of dopamine (DA) and its metabolites, dihydroxyphenyl-acetic acid (DOPAC) and homovanillic acid (HVA) in striata 7 days after MPTP treatment. As shown in Fig. 6F, MPTP injection led to a >75% reduction of the striatal DA levels compared with the striata of saline injected control mice. Interestingly, MPTP plus diapocynin treated mice showed only <40% decrease of the striatal dopamine levels (Fig. 6F). Diapocynin treatment also restored DOPAC and HVA levels significantly in MPTP treated mice (Fig. 6G &H). Next, to address whether diapocynin at a dose of 300mg/kg/day is toxic to mice, we treated mice with only diapocynin for 12 days and checked locomotor activities and striatal neurotransmitter levels. Diapocynin did not have any effect on behavioral (data not shown) or striatal dopamine content (Fig. 6F-H). In order to determine whether diapocynin interferes with the toxic metabolic conversion of MPTP to MPP⁺ by MAO-B, we measured the level of MPP⁺ in striatum 3 h after the final MPTP injection. We found that diapocynin had no effect on striatal levels of MPP⁺ (MPTP mice, 1860 ± 569 ng/g; MPTP plus diapocynin mice, 1775 ± 586 ng/g). Together, these results suggest that diapocynin is neuroprotective in MPTP mouse model of PD.

Post treated diapocynin rescues striatal neurotransmitter depletion in MPTP treated mice.
Normally after the diagnosis of the disease, patients are treated with drugs. We also speculated whether diapocynin post-treatment could protect against the MPTP-induced dopaminergic toxicity. Mice were treated with MPTP at a dose of 25mg/kg/day for 3 days followed by co-treatment of MPTP and diapocynin (300mg/kg/day) for 2 days. Mice also received another 6 doses of diapocynin (300mg/kg/day) and on 7th day mice were sacrificed for neurotransmitter analysis. As evident from Fig. SI 9, we observed significant reduction of striatal DA (~75%), DOPAC (~73%) and HVA (~70%) in MPTP treated mice. However, in MPTP treated mice that also received diapocynin 3 days after initiation of the disease, showed a reduction of ~52% of DA (Fig. SI 9A), ~50% of DOPAC (Fig. SI 9B) and ~40% of HVA (Fig. SI 9C). Thus diapocynin is capable of slowing down the progression of dopaminergic neurodegeneration in the MPTP mouse model of PD.

Discussion:

Growing evidence suggests the important role of neuroinflammation and oxidative stress in the progressive loss of nigral dopaminergic neurons in PD. However, no effective therapy is available to halt the progression of the disease. Therefore, synthesizing a novel compound with anti-inflammatory and anti-oxidative properties and testing its efficacy in a preclinical animal model of PD is essential prior to testing in humans. NADPH oxidase has emerged as a major source of oxidative stress in the brain, particularly in neurodegenerative disorders such as PD, Alzheimer’s disease, amyotrophic lateral sclerosis (ALS), and multiple sclerosis (Bedard and Krause, 2007). Apocynin is an efficient inhibitor of NADPH oxidase that generates reactive oxygen species (ROS) during the inflammatory processes (Anantharam et al., 2007). Although the mechanism of inhibition of apocynin is not clear, it
is thought to prevent the recruitment of cytosolic NADPH oxidase subunit p47phox to the membrane, thereby, inhibiting NADPH oxidase activity. Apocynin has been shown to attenuate superoxide formation and oxidative stress in vivo along with reduction of acute inflammation in lung and spinal cord (Impellizzeri et al., 2011; Impellizzeri et al., 2010; Hougee et al., 2006). In addition to that, apocynin administered at a dose of 300mg/kg/day protects against oxidative damage induced by cerebral ischemia (Wang et al., 2006) and ALS (Harraz et al., 2008). In vitro studies in dopaminergic neuronal cell lines and primary cultures also demonstrated protective role of apocynin in 1-methyl-4phenyl-pyridinium ion (MPP+) or MPTP induced NADPH oxidase mediated apoptotic cell death (Gao et al., 2003b; Anantharam et al., 2007). In contrast to anti-oxidative role of apocynin, pro-oxidative nature of apocynin has been shown in non-phagocytic cells, where it increases ROS production significantly (Vejrazka et al., 2005). Apocynin also failed to show any improvement in transgenic mouse model of AD (Dumont et al., 2011) or ALS (Trumbull KA et al., 2012). Thus, these studies suggest that the development of an improved apocynin related compound may yield a better neuroprotective agent for treatment of PD.

In this study, we chemically synthesized and delineated the functions of diapocynin, a metabolite of apocynin with potential anti-oxidative and anti-inflammatory properties. Recent studies have shown conversion of apocynin to diapocynin (apocynin dimer) in vivo which prevents the assembly and activation of NADPH oxidase complex (Johnson et al., 2002). Additionally, diapocynin is 13-fold more lipophilic than apocynin (Luchtefeld et al., 2008). Here, we show that diapocynin inhibits MPTP-induced activation and expression of both iNOS and gp91phox in activated microglia, suggesting that diapocynin is anti-
inflammatory. Diapocynin also attenuates the formation of ONOO- and 4-HNE in dopaminergic neurons in response to various stimuli, suggesting its antioxidant properties. In addition, diapocynin also protects against MPTP-induced motor deficits, striatal neurotransmitter depletion and nigrostriatal degeneration. To our knowledge, this is the first report showing anti-inflammatory, anti-oxidative stress and neuroprotective role of a novel apocynin derivative in MPTP animal model of Parkinson’s disease.

In the central nervous system, glial activation involving astrocytes, microglial cells, lymphocyte infiltration, production of proinflammatory mediators, including cytokines, chemokines, prostaglandins, reactive mediators such as RNS and ROS are all hallmarks of inflammatory reactions. Active metabolite of MPTP, MPP+ is believed to be responsible for glial activation mediated inflammation and neurodegeneration (Dauer and Przedborski, 2003). In our study, we also observed marked activation of microglia and astrocytes by western blotting and immunohistochemistry after MPTP treatment in substantia nigra and diapocynin significantly attenuated MPTP induced microgliosis and astrogliosis (Fig. 1B-G). Nuclear factor kappa B (NF-κB), a transcription factor has been shown to be an important regulator of the microglial and astroglial proinflammatory reactions in the SN. The promoter regions of proinflammatory molecules including inducible nitric oxide synthase (iNOS) contain the binding sites for NF-κB (Ghosh et al., 2007). Astroglia and microglia in the healthy brain do not express iNOS but following toxic, or inflammatory damage, reactive astroglia and microglia express iNOS in the brain (Galea et al., 1992). Nitric oxide (NO) generated by iNOS and derived from activated glial cells is believed to contribute to neurodegenerative diseases such as AD, PD, HIV associated dementia, HD and ALS (Galea
et al., 1992; Ghosh et al., 2009). Studies have shown that MPTP treatment produces significantly reduced neuronal loss in mice deficient in iNOS compared to their wild type counterparts (Liberatore et al., 1999). In this study we demonstrate that diapocynin, pharmacological inhibitor of microglial NADPH oxidase effectively attenuates MPTP-induced increases in iNOS expression (Fig. 2) suggesting the potential use of diapocynin as an anti-inflammatory agent.

Along with reactive nitrogen species (RNS), reactive oxygen species (ROS) also plays a pivotal role in oxidative stress and inflammation in PD. NADPH oxidase is the major ROS producing enzyme of microglial cells in response to various inflammatory and degenerative stimuli. NADPH oxidase is a multimeric protein composed of two membrane-bound subunits, gp91phox and p22phox, three cytosolic subunits, p47phox, p40phox, p67phox and a small GTPase rac2. It generates superoxide (O$_2^-$) from molecular oxygen. During its activation, its cytosolic subunits translocate to membrane and form the functional enzyme to generate (O$_2^-$) (Bokoch and Knaus, 2003). Neurons located very close to activated microglial cells may thus have their cell membrane proteins and lipids vulnerable to NADPH oxidase mediated superoxide and other oxidants such as hydrogen peroxide (H$_2$O$_2$). Additionally, NADPH oxidase not only mediates superoxide production, but also controls the levels of other pro-inflammatory neurotoxic factors such as TNFα and IL-1β (Qin et al., 2004). In our study, we demonstrate that diapocynin attenuated MPTP induced expression of microglial gp91phox in substantia nigra and thereby reduced the production of reactive oxygen species (Fig. 3).
Besides having direct toxic effect on nigral dopaminergic neurons, nitric oxide (NO) and superoxide (O$_2^-$) derived from astrocytes and microglia can react to form highly reactive nitrogen species peroxynitrite (ONOO$^-$). Peroxynitrite causes nitration of tyrosine residues in various proteins including tyrosine hydroxylase (TH) and $\alpha$-synuclein (Ara et al., 1998; Blanchard-Fillion et al., 2001). Peroxynitrite mediated nitration of TH is associated with reduced enzymatic activity. 3-nitrotyrosine (3-NT) is widely used as a marker of peroxynitrite in cells. Here, we found increased expression of 3-NT in dopaminergic neurons in SN of MPTP treated mice and was found to be predominantly co-localized with TH positive dopaminergic neurons (Fig. 4A-C). However, diapocynin significantly decreased MPTP-induced increased amount of 3-NT in dopaminergic neurons in the SN. Along with peroxynitrite, levels of 4-hydroxynonenol (4-HNE), an unsaturated aldehyde generated during lipid peroxidation was also significantly increased in the SN of PD brains compared to control (Yoritaka et al., 1996). The 4-HNE has been demonstrated to block mitochondria respiration and induce caspase dependent apoptosis (Picklo et al., 1999; Liu et al., 2000). In our study, we showed increased expression of 4-HNE, a marker of oxidative damage in the SN of MPTP-treated mice and it was colocalized in the cytosol of TH positive dopaminergic neurons (Fig 4D-F). Whereas, diapocynin significantly decreased the amount of 4-HNE in the MPTP-treated SN, indicating that diapocynin acted by attenuating primary production of superoxide (OH$^-$).

At present, no useful therapy of PD is available. Administration of a dopamine agonist or levodopa has been the widely used treatment for PD symptoms, but does not affect the disease pathogenesis. Dopaminergic neuroprotection in animal models of PD have been
demonstrated with various substances including glial cell line derived neurotrophic factor (GDNF), brain derived neurotrophic factor (BDNF), TGF-β (Date et al., 1993; Kordower et al., 2000). Additionally, various anti-inflammatory agents such as NSAIDs, COX inhibitors, statins, pioglitazone, minocycline have been used in different animal models of PD (Chen et al., 2003; Teismann et al., 2003; Ghosh et al., 2009; Kim and Suh, 2009). However, most of these compounds failed in either preclinical trials or in human phase I trials due to their ineffectiveness to cross the blood-brain barrier or due to limited bioavailability. Moreover, they also cause side effects and toxicity to the animals. Hence, understanding the mechanism of the disease process and development of successful neuroprotective therapeutic approach to halt the disease progression are of principal importance in the PD research field. MPTP causes glial activation and inflammation within 48h of its administration and initiates a self-perpetuating dopaminergic neurodegenerative process beginning 72h (Ghosh et al., 2009). In this study, we demonstrate diapocynin inhibits the glial cells mediated production of proinflammatory molecules, oxidative stress and protects against MPTP-induced dopaminergic degeneration in animal models of PD. Our conclusion is based on the following: (1) MPTP intoxication led to the stimulation of activation of various proinflammatory molecules within the activated glial cells in the nigra of MPTP-intoxicated mice. However, cotreatment with diapocynin resulted in attenuation of expression of proinflammatory molecules. (2) diapocynin was capable of suppressing oxidative stress in vivo in the SN of MPTP-treated mice. (3) cotreatment with diapocynin improved MPTP induced behavioral imparities. (4) diapocynin protected TH-positive dopaminergic neurons from MPTP toxicity and restored the level of dopamine and its metabolites. (5) oral administration of diapocynin on day 4, after the disease has been initiated by MPTP also
restored the levels of striatal dopamine neurotransmitters in MPTP treated mice, suggesting that diapocynin could attenuate disease progression. There are several advantages of diapocynin compared to existing anti-PD therapies including its parent compound apocynin. First, diapocynin is fairly nontoxic, mice treated with diapocynin alone (300mg/kg/day) for 12 days didn’t show any sign of behavioral imparities (data not shown), as well as their neurotransmitter levels were as per with saline treated control mice (Fig. 6F-H). Second, diapocynin can be administered by gavage-orally, the least painful route. Here we have shown that oral administration of diapocynin protects the nigrostriatum in subacute MPTP models of PD. Third, being a lipophilic molecule, diapocynin easily crosses the blood-brain barrier and enters into substantia nigra (> 1.5µg/mg tissue) and striatum (> 0.9µg/mg tissue) regions of brain as detected by LC-MS/MS (Fig. SI 7).

In summary, we have demonstrated that diapocynin (a metabolite of apocynin) attenuates nigral activation of microglial and astroglial cells, inhibits proinflammatory molecule (iNOS) and production of NADPH oxidase mediated superoxide formation, decreases oxidative stress, improves behavioral functions and protects nigrostriatum in MPTP-treated mice. Together, these results strongly suggest that diapocynin may be used as an effective anti-inflammatory, anti-oxidative stress and neuroprotective agent in therapeutic intervention of PD.

References:
(MPP+)-induced oxidative stress and apoptosis in mesencephalic dopaminergic neuronal cells. Neurotoxicology 28:988-997.


following intrastriatal injection of basic fibroblast growth factor in relation to aging.


Jin H, Kanthasamy A, Ghosh A, Yang Y, Anantharam V, Kanthasamy AG alpha-Synuclein negatively regulates protein kinase Cdelta expression to suppress apoptosis in
dopaminergic neurons by reducing p300 histone acetyltransferase activity. J Neurosci 31:2035-2051.


Figure 1

A

\[ \text{MPTP} \rightarrow \text{Diapocynin} \]

\(-1\text{d}) \rightarrow (0\text{d}) \rightarrow (1\text{d}) \rightarrow (2\text{d}) \rightarrow (3\text{d}) \rightarrow (4\text{d}) \rightarrow (8\text{d}) \rightarrow (10\text{d})

- Glial activation, inflammatory and oxidative stress markers
- Behavioral tests
- Neurotransmitter analysis

B

IBA-1

Control  \[ \text{MPTP} \]  \[ \text{MPTP + Diapocynin} \]

C

IBA-1

Control  \[ \text{MPTP} \]  \[ \text{MPTP + Diapocynin} \]

\(\beta\)-actin

D

Band intensities

E

GFAP

Control  \[ \text{MPTP} \]  \[ \text{MPTP + Diapocynin} \]

F

GFAP

Control  \[ \text{MPTP} \]  \[ \text{MPTP + Diapocynin} \]

\(\beta\)-actin

G

Band intensities

**  *
Figure 1. Diapocynin inhibits activation of microglia and astrocytes in substantia nigra (SN) of MPTP treated mice. (A) Treatment schedule of MPTP-injected mice with diapocynin. Mice were administered diapocynin (300mg/kg/day) by oral gavage 24 h before MPTP treatment and continued for 4 -5 days of co-treatment with 25mg/kg/day MPTP administered via i.p. Control mice received 10% ethanol in saline. Twenty-four h to 7 days after the last dose of MPTP, mice were sacrificed. (B) Representative western blots illustrating the expression of IBA-1 (marker of microglia) in SN. (C) Bar graph showing means Western blot IBA-1/β-actin ratios ± SEM in SN of 6 mice per group. (D) DAB immunostaining of IBA-1 in SN. (E) Representative western blots illustrating the expression of GFAP (marker of astrocytes) in SN. (F) Bar graph showing mean Western blot GFAP/β-actin ratios ± SEM in SN of 6 mice per group. (G) DAB immunostaining of GFAP in SN region of ventral midbrain. Images were captured at 30X magnification. **, p < 0.01 vs. the control group; *, p < 0.05 vs. the MPTP group.
Figure 2

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Figure 2. Diapocynin attenuates iNOS expression in substantia nigra (SN) of MPTP treated mice. Mice were administered diapocynin (300mg/kg/day) by oral gavage 24 h before MPTP treatment and continued for 4 days of co-treatment with MPTP at a dose of 25mg/kg/day. Control mice received 10% ethanol in saline. Twenty-four h after the last dose of MPTP, mice were sacrificed. (A) Representative western blots illustrating the expression of iNOS in SN. (B) Bar graph showing means Western blot iNOS/β-actin ratios ± SEM in SN of 6 mice per group. (C) Double labeling of GFAP and iNOS and (D) IBA-1 and iNOS in SN region of ventral midbrain. Images were captured at 30X magnification. ***, p < 0.001 vs. the control group; *, p < 0.05 vs. the MPTP group.
Figure 3. Diapocynin attenuates NADPH oxidase mediated inflammatory responses in SN of MPTP-treated mice. Mice were administered diapocynin (300mg/kg/day) by oral gavage 24 h prior MPTP treatment and continued for 4 days of co-treatment with 25mg/kg/day MPTP. Control mice received 10% ethanol in saline. Twenty-four h after the last dose of MPTP, mice were sacrificed. (A) Representative western blots illustrating the expression of gp91phox (membrane bound subunit of NADPH oxidase) in SN. (B) Bar
graph showing means Western blot gp91phox/β-actin ratios ± SEM in SN of 6 mice per group. (C) Substantia nigra tissue sections were double labeled for gp91phox and IBA-1. Images were captured at 20X and 60X (inserts) magnification. The substantia nigra zone is outlined in white dots. Inset pictures demonstrated colocalization of IBA-1 and gp91phox. ***, p < 0.001 vs. the control group; *, p < 0.05 vs. the MPTP group.
Figure 4

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Band intensities 3-NT/β-actin

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TH

3-NT

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Band intensities 4-HNE/β-actin

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TH

4-HNE

Hoechst

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TH

4-HNE

Hoechst

Merge

TH

4-HNE

Hoechst

Merge
Figure 4. Diapocynin inhibits the formation of 3-nitrotyrosine (3-NT) and 4-hydroxynonenol (4-HNE) in SN of MPTP treated mice. Mice were administered diapocynin (300mg/kg/day) by oral gavage 24 h before MPTP treatment and continued for 4 days of co-treatment with 25mg/kg/day MPTP via i.p. Control mice received 10% ethanol in saline. Twenty-four h after the last dose of MPTP, mice were sacrificed. (A) Representative western blots illustrating the expression of 3-NT in SN. (B) Bar graph showing means Western blot 3-NT/β-actin ratios ± SEM in SN of 6 mice per group. (C) Double labeling of TH (marker of dopaminergic neurons) and 3-NT in SN region of ventral midbrain. (D) Representative western blots illustrating the expression of 4-HNE in SN. (B) Bar graph showing means Western blot 4-HNE/β-actin ratios ± SEM in SN of 6 mice per group. (C) Double labeling of TH and 4-HNE in SN region of ventral midbrain. Images were captured at 60X magnifications. The substantia nigra zone is outlined in white dots. Inset pictures demonstrated colocalization of TH and 3-NT/4-HNE. ***, p < 0.001 vs. the control group; **, p < 0.01 vs. the control group; *, p < 0.05 vs. the MPTP group; #, p < 0.001 vs. the MPTP group.
Figure 5. Diapocynin improves motor function in MPTP injected mice. Mice were administered diapocynin (300mg/kg/day) by oral gavage 24 h before MPTP treatment and continued for 5 days of co-treatment with MPTP (25mg/kg/day) and 4 days of post-treatment with MPTP (25mg/kg/day). Control mice received 10% ethanol in saline. 4 days after the last dose of MPTP, mice were tested for motor activities. (A) VersaPlot map showing moving track of mice. VersaMax data showing (B) horizontal activity; (C) vertical activity;
(D) distance travelled (cm.); (E) movement time (sec.); (F) rest time (sec.); (G) no. of stereotypy ; (H) no. of rearing and (I) time spent on rotarod (second) at 20 rpm rod speed. Data are means ± SEM of eight to ten mice per group. ***, $p < 0.001$ vs. the control group; **, $p < 0.01$ vs. the MPTP group; *, $p < 0.05$ vs. the MPTP group; #, $p < 0.01$ vs. the control group.
**Figure 6. Diapocynin protects nigrostriatum in MPTP treated mice.** Mice were administered diapocynin (300mg/kg/day) by oral gavage 24 h before MPTP treatment and continued for 5 days of co-treatment with MPTP (25mg/kg/day) and 6 days of post-treatment of MPTP (25mg/kg/day). Control mice received 10% ethanol in saline. (A) 7 days after the last MPTP injection, mice were sacrificed and Tyrosine hydroxylase (TH)-DAB immunostaining in striatum and substantia nigra regions were performed (2X magnifications). (B) Representative western blots illustrating the expression of TH in SN and striatum. (C & D) Bar graphs showing mean Western blot TH/β-actin ratios ± SEM in SN and striatum of 6 mice per group. (E) Stereological counts of TH-positive dopaminergic neurons in the SN of ventral midbrain. 7 days after the last MPTP treatment, striatal (F) dopamine, (G) DOPAC and (H) HVA were measured by HPLC. Data are means ± SEM of eight to ten mice per group. Images were captured at 2X magnification. ***, p < 0.001 vs. control; **, p < 0.01 vs. MPTP; *, p < 0.05 vs MPTP; a, p < 0.01 vs. MPTP; #, p < 0.001 vs. MPTP.
Figure 1. Quantification of diapocynin in SN and striatum of mice. Mice were administered diapocynin (300mg/kg/day) by oral gavage 24 h before MPTP treatment and continued for 5 days of co-treatment with MPTP (25mg/kg/day) and 6 days of post-treatment of MPTP (25mg/kg/day). Seven days after last injection of MPTP, SN and ST were dissected out and quantified for diapocynin. (A) HPLC peak of diapocynin at Retention time...
1.9 min.  (B) LC-MS peak of diapocynin at retention time 1.9 min coinciding with HPLC peak.  (C) The mass spectral peak m/z 313.9 negative ion produced by diapocynin, eluting at 1.9 min.  (D) Standard curve of diapocynin standards ranging from 0.3µg to 30µg.  (E) Quantification of diapocynin in SN and ST. N=6.
Figure 2. Diapocynin protects dopaminergic neurons in MPTP model of PD. Mice were administered diapocynin (300mg/kg/day) by oral gavage 24 h before MPTP treatment and continued for 5 days of co-treatment with MPTP (25mg/kg/day) and 6 days of post-treatment of MPTP (25mg/kg/day). Control mice received 10% ethanol in saline. Seven days after the last MPTP injection, mice were sacrificed and substantia nigra sections were processed for TH (A). Double labeling of TH and FluoroJade –b in substantia nigra sections (B). TH-DAB pictures were captured at 10X magnification and TH and Fluorojade-b double labeled pictures were captured at 20X magnification.
SI figure 9. **Diapocynin suppresses disease progression in the MPTP mouse model.** Mice were treated with MPTP (25mg/kg/day) for 5 days. Diapocynin (300mg/kg/day) treatment started on 4th day of MPTP injection and continued for another 7 days. Mice were sacrificed 1 day after the last dose of diapocynin and striatal (A) dopamine, (B) DOPAC and (C) HVA were measured by HPLC. Data are means ± SEM of six to eight mice per group. ***, p < 0.001 vs. the control group; **, p < 0.01 vs. the MPTP group; *, p < 0.05 vs. the MPTP group;
CHAPTER V

NOVEL COMPOUND MITO-APOCYNIN PROTECTS DOPAMINERGIC NEURONS VIA ATTENUATING INFLAMMATION AND OXIDATIVE STRESS IN AN EXPERIMENTAL MOUSE MODEL OF PARKINSON’S DISEASE

A manuscript to be submitted to *The Journal of Immunology*

Anamitra Ghosh¹, Colleen Hogan¹, Arthi Kanthasamy¹, Vellareddy Anantharam¹, Balaraman Kalyanaraman² and Anumantha G. Kanthasamy¹

¹ Department of Biomedical Sciences, Iowa Center for Advanced Neurotoxicology, Iowa State University, Ames, IA, ² Department of Biophysics, Medical College of Wisconsin, Milwaukee, WI

Abstract:

Parkinson’s disease (PD) is one of the most common neurodegenerative disorders marked by progressive increase in non-motor and motor deficits. Recent evidences indicate that glial cell activation and its inflammatory response may contribute to the progressive degeneration of dopaminergic neurons in PD. Despite intense investigation, no neuroprotective therapy that successfully intervenes in the progression of the disease is currently available. Apocynin has been shown to possess anti-inflammatory and antioxidant properties but the clinical utility of apocynin as a neuroprotective agent is still controversial. In this present study, we investigated whether mitoapocynin, a mitochondria targeted novel derivative of apocynin, promotes the survival of dopaminergic neurons in the 1-methyl-4-
phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD. MPTP induced degeneration of dopaminergic neurons and loss of dopamine in the nigrostriatal region of midbrain. Additionally, MPTP led to activation of glial cells visualized by IBA-1 and GFAP immunostaining. Westernblotting and immunoreactivity also demonstrated increased expression of proinflammatory cytokines and molecules along with microglial NADPH oxidase which led to ROS/RNS production and formation of unsaturated aldehyde in the substantia nigra. However, oral administration with mito-apocynin improved mice behavioral activities, prevented nigrostriatal dopaminergic neurodegeneration, increased striatal dopamine. This neuroprotection afforded by mito-apocynin was associated with the attenuation of gliosis, proinflammatory molecules and cytokines, NADPH oxidase and iNOS derived RNS production and lipid peroxidation. Collectively, our results demonstrate that mitoapocynin produces distinct anti-inflammatory and antioxidant effects in a well-established neurotoxicity animal model of PD. These data strongly suggest that additional preclinical development of mitoapocynin may yield an effective neuroprotective drug capable of intervening in the progression of Parkinson’s disease.

Introduction

Parkinson’s disease (PD) is a common neurodegenerative movement disorder characterized by motor symptoms such as retig tremor, rigidity, bradykinesia and postural instability along with several non-motor symptoms (Dauer and Przedborski, 2003; Vila and Przedborski, 2004). The pathological hallmark of PD is the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) of the midbrain that project to the striatum. Parkinsonian symptoms appear when majority (60 or 70%) of SNpc dopaminergic neurons
are lost, resulting in depletion of nigrostratal dopamine. The disease mechanism that causes the disease is not completely known, but recent evidences suggest that glial cells mediated inflammation and oxidative stress increase the risk of developing PD (Dauer and Przedborski, 2003). Sustained microglial and astroglial activation in close proximity of dying dopaminergic neurons in the SNpc is evident in PD patients and animal models of the disease (McGeer et al., 1988; Hirsch and Hunot, 2009). Different in vitro and in vivo animal models have demonstrated elevation of the key enzymes involved in the production of reactive oxygen species (ROS) or reactive nitrogen species (RNS), such as microglial NADPH oxidase and inducible nitric oxide synthase (iNOS) and astroglial myeloperoxidase (MPO) in dopaminergic neurodegenerating areas of substantia nigra (SN) (Gao et al., 2003; Wu et al., 2003). In addition to that, variety of pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-α), interleukin (IL) -1beta (β), IL-6, interferon gamma (IFN-γ) and other immune neurotoxins are found either in CSF or affected regions of PD brain (Nagatsu et al., 2000). However, the mechanism behind glial cells activation in the diseased state is not completely known. A recent study has shown that the activation of NF-κB in microglial and astroglial cells play critical neurotoxic role by mediating proinflammatory cytokine levels in the SN (Ghosh et al., 2007). Nurr1, a nuclear orphan receptor suppresses glial activation by docking to NF-κB -p65 on target inflammatory gene promoters and protects against dopaminergic neurodegeneration in SN (Saijo et al., 2009). Collectively, these findings suggest controlling inflammation and oxidative stress could be a key neuroprotective strategy in PD and its animal model.

Along with inflammation and oxidative stress, PD is also associated with mitochondrial dysfunction leading to energy failure (Betarbet et al., 2000). Defects in
complexes I, II, and IV of the mitochondrial respiratory chain have been detected in neurodegenerative disorders such as Alzheimer's, Parkinson's, and Huntington's diseases. Various anti-oxidants and chelators have been tested in different animal models of PD with little success (Kaur et al., 2003; Golden and Patel, 2009). In our study we synthesized a novel mitochondria targeted anti-oxidant mito-apocynin which is also a derivative of apocynin and checked its anti-oxidant and anti-inflammatory roles in MPTP mouse model of PD. Here in, we demonstrate that in primary culture, mito-apocynin blocks MPP⁺-induced dopaminergic neurodegeneration by attenuating inflammatory reactions and oral administration of mito-apocynin in mice protects the dopaminergic neurons and terminals from MPTP toxicity by reducing glial activation and glial cells mediated inflammatory reactions and oxidative stress. Collectively, these results strongly suggest that additional preclinical development of mito-apocynin may yield an effective neuroprotective drug for treating PD patients.

Materials and methods:

Chemicals and biological reagents. 1-Methyl-4-phenyltetrahydropteridine (MPP⁺ iodide) and MPTP-HCl were purchased from Sigma (St. Louis, MO, USA). RPMI, neurobasal medium, B27 supplement, fetal bovine serum, L-glutamine, Sytox assay dye, IR-dye tagged secondary antibodies, penicillin, and streptomycin and other cell culture reagents were purchased from Invitrogen (Gaithersburg, MD). Recombinant murine TNF, LPS (E. coli 0111:B4) and cytosine arabinoside were purchased from Sigma-Aldrich (St. Louis, MO).

Mesencephalic Primary Neuron Cultures and Treatment. Primary mesencephalic culture was prepared from the ventral mesencephalon of gestational 14- to 15-day-old mice embryos as described previously. Briefly, mesencephalic tissues from E14 to 15 mouse embryos were
dissected and maintained in ice-cold calcium-free Hanks’ balanced salt solution and then dissociated in Hanks’ balanced salt solution containing trypsin-0.25% EDTA for 20 min at 37°C. The dissociated cells were then plated at equal density of 0.6 million cells per well on 12-mm coverslips precoated with 0.1 mg/ml poly-D-lysine. Cultures were maintained in neurobasal medium fortified with B-27 supplement, 500 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA). The cells were maintained in a humidified CO₂ incubator (5% CO₂ and 37°C) for 24 h. Half of the culture medium was replaced every 2 days. Approximately 6- to 7-day-old cultures were used for experiments. Primary mesencephalic and striatal dopaminergic neuronal cells were exposed to 10 μM MPP⁺ in the presence or absence of mito-apocynin (10 μM) for 24 h.

**Primary microglial cultures and treatments.** Primary microglial and astroglial cultures were prepared from C57BL/6 postnatal day 1 (P1) mouse pups. The mouse brains were harvested, meninges removed, and then placed in Dulbecco’s modified Eagle’s medium/F-12 nutrient media (DMEM-F12, GIBCO Cat # 11320) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 U/mL penicillin, 50 μg/mL streptomycin, 2 mM L-glutamine, 100 μM non-essential amino acids, and 2 mM sodium pyruvate (Invitrogen). The brain tissue was then incubated in 0.25% trypsin for 30 min with gentle agitation. The trypsin reaction was stopped by adding an equal volume of DMEM/F12 complete medium and the brain tissue was washed 3 times. The tissue was then triturated gently to prepare a single cell suspension and then passed through a 70 μm nylon mesh cell strainer to remove tissue debris and aggregates. The cell suspension then was made up in DMEM/F12 complete medium and seeded into T-75 flasks. The cells were placed in humidified CO₂ incubator at 37°C, the medium was changed after 4 to 5 days and the mixed glial cells were grown to confluence.
Microglial cells were separated from confluent mixed glial cultures by differential adherence and magnetic separation to >97% purity as described in our recent publication (Ghosh et al., 2010). Both microglia and astrocytes were allowed to recover for at least 48 hours after plating. Primary microglia and astrocytes were treated in DMEM/F12 complete medium containing 2% FBS.

**Microglial nitric oxide detection.** Nitric oxide production by primary microglia culture was measured indirectly by quantification of nitrite in the supernatant using the Griess reagent (Sigma Aldrich). BV2 microglial cells were plated in poly-D-lysine coated 96-well plates at 50,000 cells per well. Cells were treated with either 1µg/ml LPS for 24 h in presence or absence of different doses of mito-apocynin and 100 μL of supernatant was collected from each well and an equal volume of the Griess reagent was added. The samples were incubated on a plate shaker at room temperature for 15 min until a stable color was obtained. The absorbance at 540 nm was measured using a Synergy 2 multi-mode microplate reader (BioTek Instruments) and the nitrite concentration determined from a sodium nitrite standard curve.

**Multiplex cytokine and chemokine Luminex immunoassays.** Primary microglia from time pregnant pups from C57BL/6 mice were obtained and seeded in poly-D-lysine coated 96-well plates respectively. The cells were treated for 24 h with 100 ng/mL LPS in the presence or absence of different doses of mito-apocynin. After treatment, 50 μL of supernatant from each well was collected and frozen at -80°C. The levels of cytokines and chemokines in the supernatants were determined using the Luminex bead-based immunoassay platform (Vignali, 2000) using pre-validated multiplex kits (Milliplex mouse cytokine and chemokine panels – Millipore Corporation) according to the manufacturer’s instructions.
**Intracellular reactive oxygen species (iROS) detection.** Intracellular reactive oxygen species (iROS) were determined using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Calbiochem), according to previously published reports (Zhang et al., 2005; Qian et al., 2007). Magnetically separated microglia from C57 black mice were plated at $10^5$ cells per well in 96-well plates and allowed to attach overnight. Before treatment, cells were washed in HBSS and incubated with 40 µM DCFH-DA in HBSS containing 2% FBS for 30 min. Cells were then treated with 100 ng/mL LPS for 24h in the presence or absence of different doses of mito-apocynin. The fluorescence intensity was determined using a Synergy-2 multi-mode microplate reader at an excitation of 485/20 nm and an emission of 530/25 nm. The fluorescence value from the control cultures was subtracted as background and the increase in fluorescence with treatments was expressed as increased iROS as previously described (Zhang et al., 2005; Qian et al., 2007).

**Immunocytochemistry.** The primary mesencephalic and astroglial cultures were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 20 minutes and processed for immunocytochemical staining as described previously (Ghosh et al., 2010). First, nonspecific sites were blocked with 2% bovine serum albumin, 0.5 % Triton X-100 and 0.05% Tween-20 in PBS for 45 min at room temperature. Cells were then incubated with different primary antibodies such as TH (1:1600, mouse monoclonal; Millipore, Billerica, MA), GFAP (1:1000, mouse monoclonal; Chamicon), p65 (1:500, rabbit polyclonal; Santa Cruz) at 4°C overnight. Appropriate secondary antibodies (Alexa Fluor 488 or 594 or 555; Invitrogen) were used followed by incubation with 10µg/ml Hoechst 33342 (Invitrogen) for 5 min at room temperature to stain the nucleus. The coverslip containing stained cells were washed twice with PBS and mounted on poly-D lysine coated slides (Sigma, St Louis). Cells were
viewed under a NIKON inverted fluorescence microscope (model TE-2000U; NIKON, Tokyo, Japan) and images were captured with a SPOT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

**Animals and treatment.** Eight-week-old male C57BL/6 mice weighing 24 to 28 g were housed in standard conditions: constant temperature (22 ± 1°C), humidity (relative, 30%), and a 12-h light/dark cycle. Use of the animals and protocol procedures were approved and supervised by the Committee on Animal Care at Iowa State University (Ames, IA). Mice received mito-apocynin (3mg /kg / day) in 10% ethanol by oral gavage either 1 day before or after 3 days of the MPTP insult. For MPTP treatment, mice received 25mg/kg/day MPTP-HCl in saline intraperitoneally for consecutive 5 days. Control mice received equivolume injection of 1XPBS.

**HPLC analysis of striatal dopamine and its metabolites levels.** Samples were prepared and quantified as described previously (Ghosh et al., 2010). In brief, 7 days after MPTP injection mice were sacrificed and striata were collected and stored at -80°C. On the day of analysis, neurotransmitters from striatal tissues were extracted using an antioxidant extraction solution (0.1 M perchloric acid containing 0.05% Na$_2$EDTA and 0.1% Na$_2$S$_2$O$_5$) and isoproterenol (as internal standard). DA, DOPAC and HVA were separated isocratically by a reversed-phase column with a flow rate of 0.6 ml/min. An HPLC system (ESA Inc., Bedford, MA) with an automatic sampler equipped with refrigerated temperature control (model 542; ESA Inc.) was used for these experiments. The electrochemical detection system consisted of a Coulochem model 5100A with a microanalysis cell (model 5014A) and a guard cell (model 5020) (ESA Inc.). The data acquisition and analysis were performed using the EZStart HPLC Software (ESA Inc.).
qRT-PCR. After treatment, total RNA were extracted from the mice brain SN using Absolutely RNA Miniprep Kit (Agilent Technologies, Santa Clara, CA) following the manufacturer’s protocol. Total RNA was treated with DNase I to remove DNA contamination and then reversibly transcribed into first-strand cDNA using the SuperScript III first-strand synthesis system (Invitrogen, Carlsbad, CA) as described in the kit instructions. SYBR-green Quantitative PCR was performed with validated primers of TNF-α, IL-1β, iNOS, IL-6 and control 18S primers (Qiagen, Valencia, CA) using RT² SYBR® Green qPCR Master Mix (SABiosciences, Fredrick, MD) for the mice tissue.

Western blotting. Mice were sacrificed 3 days after MPTP treatment and substantia nigra tissue was dissected out. Brain lysates containing equal amounts of protein were loaded in each lane and separated in a 10 to 15% SDS-polyacrylamidegel electrophoresis gel as described previously (Jin et al., 2011). Poteins were transferred to a nitrocellulose membrane and nonspecific binding sites were blocked by treating with Licor odyssey blocking buffer. The membranes were then incubated with different primary antibodies such as anti-IBA-1 (Abcam), anti-GFAP (Chemicon), anti-iNOS (Santa Cruz), anti-gp91phox (Abcam), anti-3NT (Chemicon) and anti-4HNE (R&D). Next, membranes were incubated with Alexa Fluor 680 goat anti-mouse or Alexa Fluor 680 donkey anti-goat (Invitrogen) or IR dye 800 donkey anti-rabbit (Rockland) secondary antibodies. To confirm equal protein loading, blots were reprobed with a β-actin antibody (Sigma; 1:10000 dilution). Western blot images were captured with a Licor Odyssey machine (Licor, NE). The western blot bands were quantified using NIH Image J software.

Immunohistochemistry. Three days after last MPTP treatment, mice were perfused with 4% paraformaldehyde and post fixed with PFA and 30% sucrose respectively. Next, fixed brains
were cut into 30µm sections and were incubated with different primary antibodies such as anti-IBA-1 antibody (Abcam), anti-GFAP (Chemicon), anti-iNOS (Santa Cruz), anti-3NT (Chemicon), anti-gp91phox (Abcam) and anti-4HNE (R & D) for overnight at 4C. Appropriate secondary antibodies (Alexa Fluor 488 or 594 or 555 from Invitrogen) were used followed by incubation with 10µg/ml Hoechst 33342 for 5 min at room temperature to stain the nucleus. Sections were viewed under a Nikon inverted fluorescence microscope (model TE-2000U; Nikon, Tokyo, Japan); images were captured with a SPOT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

**DAB immunostaining and stereological counting.** DAB immunostaining was performed in striatal and substantia nigral sections as described previously (Ghosh et al., 2009; Ghosh et al.). In brief, 30 µm sections were incubated with either anti-TH antibody (Calbiochem, rabbit anti-mouse, 1:1800) or anti-IBA-1 (Abcam, goat anti-mouse, 1:1000) or anti-GFAP (Chemicon, mouse anti-mouse, 1:1000) antibody for overnight at 4C. Next, sections were incubated in biotinylated anti-rabbit or goat or mouse secondary antibody followed by incubation with avidin peroxidase (Vectastatin ABC Elite kit, Vector laboratories). Immunolabeling was observed using diaminobenzidine (DAB), which yielded a brown stain. Total numbers of TH positive neurons in SN were counted stereologically with Stereo Investigator software (MBF Bioscience, Williston, VT, USA), using an optical fractionators (Ghosh et al., 2010).

**Behavioral measurements.** An automated device (AccuScan, model RXYZCM-16, Columbus, OH) was used to measure the spontaneous activity of mice. The activity chamber was 40×40×30.5 cm, made of clear Plexiglas and covered with a Plexiglas lid with holes for
ventilation. Data were collected and analyzed by a VersaMax Analyzer (AccuScan, model CDA-8, Columbus, OH). Before any treatment, mice were placed inside the infrared monitor for 10 min daily and 5 min daily for 3 consecutive days to train them. After 5 days of last MPTP injection, openfield and rotarod experiments were conducted as described previously (Ghosh et al., 2010). Locomotor activities (horizontal activities, vertical activities, total distance travelled, total rest time and rearing activities) were presented for 10 min test session. For rotarod experiment 20 rpm speed was used. Mice were given a 5-7 min rest interval to eliminate stress and fatigue.

Data analysis. Data analysis was performed using Prism 4.0 software (GraphPad Software Inc., San Diego, CA). Raw data were first analyzed using one-way analysis of variance and then Tukey’s post-test was performed to compare all treatment groups. Differences with \( p < 0.05 \) were considered significant.

Results:

**Mito-apocynin protects dopaminergic neurons against MPP\(^+\)- induced toxicity in primary mesencephalic cultures**

We first examined whether mito-apocynin (Fig. 1) can rescue TH-positive neurons from MPP\(^+\)-induced toxicity in primary mesencephalic cultures. Primary mesencephalic cultures were isolated from E15 C57 BL/6 mice embryos, and neurons were treated with 10\( \mu \)M MPP\(^+\) in the presence or absence of 5\( \mu \)M mito-apocynin. After 24h of treatment, primary neurons were processed for TH immunochemistry. As shown in Fig. 1, MPP\(^+\) treatment induced an approximately 75% loss of TH cell count (Fig. 1C) and 85% loss of neurite processes (Fig. 1B). However, 5\( \mu \)M mito-apocynin treatment significantly protected
against MPP⁺ neurotoxicity. Mean lengths of TH⁺ve neuronal processes in MPP⁺ plus mito-apocynin treated primary neurons were significantly longer (p < 0.01) than the MPP⁺-treated processes. Similarly, mito-apocynin significantly (p < 0.05) rescued dopaminergic neurons against MPP⁺ toxicity. These results suggest that the mito-apocynin possesses neuroprotective properties in the MPP⁺-induced dopaminergic degenerative model.

**Mito-apocynin inhibits MPP⁺ induced activation of NF-κB in primary culture**

Microglial activation plays an important role in the pathogenesis of PD as well as other neurodegenerative disorders (Dauer and Przedborski, 2003; Gao et al., 2003; Ghosh et al., 2007). Neurotoxin MPTP mimics some features of PD in rodents and primates. The neurotoxic effect of MPTP depends on its conversion into MPP⁺ which then activates glial cells (Dauer and Przedborski, 2003). Recently, it has been demonstrated that MPP⁺ induces the activation of NF-κB in glial cells and promoter region of proinflammatory molecules contain the DNA binding site for NF-κB (Hayden and Ghosh, 2004; Ghosh et al., 2007). Similarly, we also observed increased NF-κB -p65 expression in primary dopaminergic neurons (Fig. 2A) and primary astrocytes (Fig. 2B) in MPP⁺ treated cultures and this p65 was present in both nucleus and cytoplasm because part of it did not colocalize with hoechst (Fig. 2A-B). However, as seen in Fig. 2A and B, mito-apocynin inhibited the expression of p65 in dopaminergic neurons and astrocytes. It also blocked the entry of p65 into the nucleus.

**Mito-apocynin inhibits LPS-induced inflammatory processes in primary microglial cells.**

After establishing inhibitory role of mito-apocynin in p65 activation, we were interested to see whether mito-apocynin could attenuate LPS-induced inflammatory processes. First, we measured the levels of pro-inflammatory cytokines from supernatant of
LPS plus mito-apocynin treated culture. Primary microglial culture from day-1-old pups were treated with 100ng/ml LPS in presence or absence of two different doses (20μM and 40μM) of mito-apocynin. 24 h after treatment, the levels of various pro-inflammatory cytokines in the supernatant were determined using a multiplex bead-based Luminex assay system (Milipore Bioplex). As evident in Fig. 3A-C, LPS triggered significant (p < 0.001) induction of TNF-α, IL-6 and IL-12 release in the supernatant, whereas, mito-apocynin treatment significantly attenuated the cytokine release.

Nitric oxide production and intracellular ROS (iROS) generation are important aspects of the microglial activation response and are particularly relevant in PD models where deregulation of microglial activation is believed to trigger dopaminergic neurodegeneration via oxidative stress. We checked whether mito-apocynin could attenuate LPS-induced nitric oxide generation in primary microglia culture. Nitric oxide production was measured indirectly using Griess assay in primary microglia (Fig. 3D). Cells were treated with LPS (100ng/ml) in the presence or absence of mito-apocynin (20μM or 40μM) for 24 h. A sharp increase in nitrite levels (2 μM; p < 0.001) of LPS-treated culture in comparison to control was observed, however, mito-apocynin dose dependently attenuated LPS-induced nitrite levels (Fig 3D). Next, we wanted to test whether mito-apocynin inhibited intracellular superoxide release in primary microglial culture. As evident in Fig. 3E, LPS treatment induced intracellular ROS levels in primary microglia at 12 h, whereas LPS plus mito-apocynin had significantly lower levels of intracellular ROS. Together, these results provide anti-inflammatory role of mito-apocynin in the regulation of different aspects of the microglial proinflammatory responses.
Mito-apocynin improves motor behavior in MPTP mice

After establishing efficacy of mito-apocynin in attenuation of inflammatory reactions and protection of dopaminergic neurons against MPP⁺ toxicity in primary culture, we examined the neuroprotective effect of mito-apocynin in a preclinical model of PD. We first evaluated whether mito-apocynin improves MPTP-induced motor deficits by testing behavioral performances in openfield and rotarod instrument as described recently (Ghosh et al., 2010). Animals receiving mito-apocynin (3mg/kg/day) were evaluated 4 days after MPTP treatment in Versamax infrared computerized activity monitoring system and rotarod instrument (Accuscan, OH). Representative motor activity maps of movement of saline treated control, MPTP and MPTP plus mito-apocynin treated mice are shown in Fig. 4A. As observed in the Versa Plot (Fig. 4A), we found decreased movement of MPTP treated mice and improvement of locomotion in MPTP plus mito-apocynin treated group. MPTP treatment decreased horizontal activity (Fig. 4B), vertical activity (Fig. 4C), total distance travelled (Fig. 4D), movement time (Fig. 4E), no. of stereotypy (Fig. 4G), no. of rearing (Fig. 4H) and time spent on rod at 20 rpm speed (Fig. 4I) consistent with our previous observations (Ghosh et al., 2010). Additionally, rest time was increased in the MPTP treated mice (Fig. 4F). However, the behavioral dysfunctions in MPTP-treated mice were recovered significantly by mito-apocynin (Fig. 4B-I).

Mito-apocynin protects nigrostriatal dopaminergic neurons and its fibers from MPTP-induced neurotoxicity in vivo

After doing behavioral experiments mice were sacrificed 7 days post MPTP treatment and brains were removed and sections were immunostained for tyrosine hydroxylase (TH) immunostaining to detect dopaminergic neurons and fibers. As observed earlier (Ghosh et al.,
2010), there was a significant loss of TH positive cell bodies and terminals in SN and striatum respectively (Fig. 5A and B) in MPTP treated mice compared with those in PBS-treated control mice (Fig. 5A and B). Higher magnified 10X pictures (Fig. 5B- lower panel) clearly demonstrated loss of neurons in substantia nigra pars compacta (SNpc), substantia nigra lateralis (SNI) and substantia nigra reticularis (SNr) regions of nigral tract of MPTP-treated mice. To investigate whether mito-apocynin altered MPTP-induced neurodegeneration in nigrostriatum, we started gavage administration of mito-apocynin (3mg/kg/day) 1 day before MPTP injection and continued for 12 days. The results of TH immunostaining showed that mito-apocynin significantly reduced MPTP-induced death of dopaminergic neurons in SN and their terminals in striatum (Fig. 5A-B). Next, to determine whether mito-apocynin protects against neurotransmitter loss induced by MPTP, we sacrificed another batch of mice 7 days after MPTP treatment and striatal dopamine, DOPAC and HVA were measured by HPLC (Fig. 5C). In parallel with dopaminergic neuronal loss evidenced by immunohistochemistry, HPLC analysis revealed 75% loss of striatal dopamine compared with the PBS treated control mice. By contrast, treatment of MPTP-treated mice with mito-apocynin (3 mg / kg / day) increased dopamine levels by 66% compared with MPTP mice (p < 0.001; Fig. 2C). In addition to dopamine, MPTP treatment also led to 76% and 70% loss of DOPAC and HVA in striatum respectively, whereas mito-apocynin treatment restored those metabolite levels significantly (Fig. 5C).

**Mito-apocynin inhibits microglial and astroglial activation in the SN in vivo**

Various evidences suggest that activated glial cells in SN play an important role in dopaminergic neurodegeneration in the human PD patients and in the MPTP model (Dauer and Przedborski, 2003; Block and Hong, 2007; Ghosh et al., 2007). Thus, we next evaluated
whether the neuroprotective effect of mito-apocynin resulted from attenuation of gliosis in the SN. At 3 day after MPTP treatment, mice were sacrificed and nigral tissue sections were processed for IBA-1 (marker of microglia) and GFAP (marker of astrocytes) immunostaining (Fig. 6A-B). We observed increased expression of activated microglial cells (characterized by amoeboid shape with thick processes) and astrocytes (characterized by bigger cell bodies with thick processes) in SN of MPTP treated mice compared with those in PBS treated control mice (Fig. 6A-B). However, mito-apocynin treatment reduced the number of IBA-1 positive microglial cells and GFAP positive astroglial cells in the MPTP-treated SN (Fig. 6A-B). To further confirm these findings, we also did western blotting of IBA-1 and GFAP in SN tissue. In MPTP-treated SN tissues, IBA-1 and GFAP expression increased significantly compared with PBS treated control samples (Fig. 6C-F). By contrast, gavage treatment with mito-apocynin significantly attenuated the expression of IBA-1 and GFAP in MPTP treated mice (Fig. 6C-F). Collectively, these results demonstrate anti-gliosis role of mito-apocynin (Fig. 6A-B).

**Mito-apocynin inhibits the expression of inducible nitric oxide synthase (iNOS) in the SN of MPTP treated mice**

Previous reports demonstrated increased level of inducible nitric oxide synthase in SN of MPTP-treated mice (Liberatore et al., 1999; Ghosh et al., 2009). Thus, we also examined whether mito-apocynin might modulate dopamine neuronal survival by affecting MPTP-induced expression of iNOS in the SN. Three days after MPTP treatment, mice were sacrificed and SN tissues were dissected out and immunoblotted for iNOS (Fig. 7A). We found marked increase in expression of iNOS in the SN of MPTP-treated mice when compared to PBS-treated control mice. However, mito-apocynin attenuated MPTP-induced
expression of iNOS protein in the SN (p < 0.05; Fig. 7A). Additionally, immunofluorescence analysis for iNOS in SN sections shows that MPTP treatment led to marked increase in nigral iNOS protein expression and that iNOS colocalized with IBA-1-positive microglia and GFAP-positive astroglial (Fig. 7B and C). However, consistent to their inhibitory effect on the activation of glial cells, mito-apocynin attenuated MPTP-induced expression of iNOS in the glial cells (Fig. 7B and C).

**Mito-apocynin inhibits the expression of proinflammatory molecules in vivo in the midbrain of MPTP-treated mice**

Glial cells when get activated release proinflammatory cytokines along with iNOS. Similarly, we also found marked increase in mRNA expression of iNOS, TNF-α, IL-1β and IL-6 in the ventral midbrain region of brain 3 days after MPTP administration (Fig. 8). However, 3mg/kg/day mito-apocynin treatment strongly inhibited MPTP-induced expression of these proinflammatory cytokines in vivo in the ventral midbrain (Fig. 8). These results strongly suggest that mito-apocynin can suppress the expression of proinflammatory molecules in vivo in the ventral midbrain of MPTP-treated mice.

**Mito-apocynin attenuates activation of NADPH oxidase in activated microglia of MPTP-treated mice**

Recent reports demonstrated that NADPH oxidase-induced oxidative stress played pivotal role in neurodegeneration of dopaminergic neurons in PD and in MPTP mouse model (Vejrazka et al., 2005). Thus, we also checked the protein expression of gp91phox, a major component of NADPH oxidase in SN region of mouse brain three days after MPTP treatment. The western blot analysis showed an increased expression of gp91phox in MPTP-
treated mice compared to weak expression in saline treated mice (Fig. 9A-B). However, mito-apocynin attenuated MPTP-induced expression of gp91phox protein in the SN (p < 0.05; Fig. 9B). Additionally, robust gp91phox immunoreactivity was seen specifically in larger cells with thick, shorter ramifications in the SN of MPTP treated mice (Fig. 9C). Double immunolabeling studies confirmed that gp91phox immunoreactivity appeared to colocalize with IBA-1 positive microglia (Fig. 9C, middle panel). Previous study suggested that in the MPTP model of PD, gp91phox does not colocalize either with astrocytes or dopaminergic neurons (Wu et al., 2003). However, mito-aocynin treatment attenuated MPTP induced gp91phox protein expression in the IBA-1 positive microglial cells in the SN (Fig. 9C). These results suggest that mito-apocynin effectively blocks the MPTP-induced microglial NADPH oxidase.

**Mito-apocynin attenuates nitrosative damages in the SN in vivo**

Nitration of protein tyrosine residues is believed to be a well known marker of oxidative stress in PD patients and its animal model (Dawson and Dawson, 2003). In oxidative stress condition, iNOS derived oxidants selectively nitrates the tyrosine residues and generates 3-nitrotyrosine (3-NT) (Ara et al., 1998). To observe the levels of iNOS-mediated oxidative damage to tyrosine residues, we checked the expression of 3-NT 3 days after MPTP treatment in presence or absence of mito-apocynin. The western blot analysis showed robust increase in expression of 3-NT protein in SN compared with those in the PBS treated control mice (Fig 10A-B). However, mito-apocynin significantly attenuated (p < 0.05; Fig 10B) MPTP-induced 3-NT expression. Consistent with this western blotting result, immunohistochemistry analysis also demonstrated increased levels of 3-NT expression in TH positive dopaminergic neurons in SN of MPTP-treated mice (yellow color cells showing
colocalization of TH and 3-NT) compared with the PBS treated SN of control mice (Fig. 10C). But, this robust increase of nitrotyrosine levels in MPTP-treated mice was dramatically inhibited by mito-apocynin, as very few TH positive cells express nitrotyrosine protein in their cytosol (Fig. 10C). Collectively, these results demonstrate that mito-apocynin is capable of reducing iNOS derived oxidative stress.

**Mito-apocynin reduces the formation of lipid peroxidation in the nigral dopaminergic neurons in vivo**

Along with peroxinitrite, lipid peroxidation also induces neuronal apoptosis in the presence of oxidative stress (Awasthi et al., 2008). 4-hydroxynonenol (4-HNE) is widely used as a marker of membrane lipid peroxidation induced by cytotoxic radicals such as hydroxyl (-OH) (Stadler et al., 2008). Previous reports demonstrated increased levels of 4-HNE in PD brain and MPTP mouse model (Selley, 1998; Fujita et al., 2009). In our study, we also observed robust increase in 4-HNE expression in SN of MPTP-treated mouse 3 days after MPTP administration compared to PBS treated control mice (Fig. 11A-B). However, mito-apocynin (3mg /kg /day) treatment significantly reduced the MPTP-induced expression of 4-HNE in SN (p < 0.01; Fig. 11B). Consistent with western blotting, we also observed increased expression of 4-HNE in SN of MPTP-treated mice and 70-80% TH-positive dopaminergic neurons express 4-HNE in their cytosol (Fig. 11C). In contrast, treatment of MPTP-treated mice with mito-apocynin resulted in reduction of 4-HNE expression in the dopaminergic neurons in the SN (Fig. 11C). Collectively, these results demonstrated that mito-apocynin is capable of reducing oxidative stress in nigral neurons in MPTP mouse model of PD.
Discussion:

In this study, we demonstrated that mito-apocynin protects against MPP+ induced loss of dopaminergic neurons in primary culture and nigrostriatal dopaminergic neurons from MPTP-induced neurotoxicity in vivo by attenuating glial cells mediated inflammatory reactions. We presented evidence that novel compound mito-apocynin blocked MPP⁺-induced activation of NF-κB, resulting in attenuation of cytokine release and oxidative and nitratative stress in primary culture. In parallel to that, MPTP-induced ROS/RNS generation, pro-inflammatory cytokine production and NADPH oxidase activation in glial cells and oxidative damage to the dopaminergic neurons were also inhibited by mito-apocynin in vivo. Depletion of these inflammatory and oxidative stress processes led to reversal of motor imparities, survival of nigrostellar dopaminergic neurons and terminals and restoration of striatal neurotransmitter levels.

In laboratory, different neurotoxins have been widely used as a compound that could induce neurochemical changes and symptoms in animals mimicking those of human PD patients (Gerlach, 1991; Vila and Przedborski, 2003). Substantial decrease in complex I to IV enzymes have been reported in PD patients (Dauer and Przedborski, 2003). Similarly, neurotoxin MPTP mimics major biochemical and pathophysiological properties of idiopathic PD by inhibiting complex I enzyme of mitochondria (Przedborski et al., 2004). Although, the MPTP animal model is the best and only available drug-induced preclinical model, its relevance to idiopathic PD is still questionable as MPTP does not mimic the progressive loss of dopaminergic neurons. Along with that, formation of lewy bodies and non motor symptoms which are very common in PD patients are also absent in this model. But, among all neurotoxins, only MPTP replicates clinical symptoms indistinguishable from idiopathic
PD. So, the MPTP model of PD is an alternate, reliable, effective rodent model for testing the effectiveness of mitochondria targeted neuroprotective agent. Many conventional antioxidant compounds (vitamin E, tempol, ubiquinone) failed to attenuate ROS/RNS in preclinical models due to their inability to accumulate in mitochondria (Murphy, 1997; Smith et al., 1999). In our study, we chemically synthesized mito-apocynin, a mitochondria targeted antioxidant with potential anti-oxidative and anti-inflammatory properties. Mito-apocynin is also a derivative of apocynin, a known NADPH oxidase inhibitor. Apocynin had been shown to attenuate superoxide formation and oxidative stress in vivo in spinal cord (Bedard and Krause, 2007). Additionally, apocynin administered at a dose of 300mg/kg/day protects against oxidative damage induced by ALS (Harraz et al., 2008). In vitro studies in dopaminergic neuronal cell lines and primary cultures also demonstrated protective role of apocynin in 1-methyl-4-phenyl-pyridinium ion (MPP⁺) or MPTP induced NADPH oxidase mediated apoptotic cell death (Anantharam et al., 2007). In contrast, apocynin increased ROS production significantly, showing its pro-oxidative nature (Vejrazka et al., 2005). Apocynin was also unable to improve behavioral and neuropathological deficits in a transgenic mouse model of AD (Gao et al., 2003). In our study we established two functions of mito-apocynin. In one hand, it attenuates glial cells mediated formation of pro-inflammatory molecules and NADPH oxidase in vitro and in vivo, suggesting its anti-inflammatory role. On the other, it inhibits formation of peroxynitrite and lipid peroxidation in dopaminergic neurons, suggesting its anti-oxidant activities. Moreover, mito-apocynin also protects against MPTP-induced motor abnormalities, striatal dopamine depletion and nigrostriatal dopaminergic degeneration.
In resting phase, microglia and astrocytes play an important role in supporting neurons in the brain and spinal cord. But, in the presence of adverse chemical or environmental stimuli they become activated and cause neuronal cell death. Activated microglia and astrocytes produce ROS/RNS and proinflammatory molecules. In our study, we also observed marked increase in glial activation in primary culture and nigrostriatum of MPTP treated mice. However, mito-apocynin reduces MPP⁺/MPTP induced glial activation (Fig. 2 & 3). Previous reports have shown that iNOS and proinflammatory cytokine such as β, IL-6 and TNF-α increased in the PD brain and in MPTP model of PD (Nagatsu et al., 2000). Moreover, nitric oxide (NO) generated by iNOS and derived from activated glial cells is believed to contribute to neurodegenerative diseases such as AD, PD, HIV associated dementia, HD and ALS (Galea et al., 1992; Ghosh et al., 2009). Proinflammatory cytokines such as TNF-α, IL-6 and IL-1β secreted from activated microglial cells may facilitates intracellular death-related signaling pathways or participate in activation of iNOS expression in the MPTP mouse model (Teismann et al., 2003). In addition to that, promoter region of iNOS and pro-inflammatory cytokines contains the DNA binding site for NF-κB, a transcription factor which regulates glial cells mediated inflammatory reactions (Hayden and Ghosh, 2004). *In vitro* data in our experiment clearly demonstrated attenuation of LPS-induced production of reactive nitrative species and reactive oxygen species in mito-apocynin treatment. Moreover, *in vivo* studies show increased expression of iNOS and proinflammatory cytokines in ventral midbrain regions of MPTP treated mice. However, oral gavage treatment of mito-apocynin inhibited MPTP-induced expression of these proinflammatory molecules in glial cells. These results strongly suggest that mito-apocynin has anti-inflammatory properties that contribute to its neuroprotective effects.
Along with iNOS mediated RNS, microglia-derived NADPH oxidase can also produce ROS and cause oxidative stress in PD patients and MPTP model of PD (Oyagi et al., 2008). NADPH oxidase is a five-subunit protein that generates superoxide from molecular oxygen and is composed of three cytosolic subunits p47phox, p40phox and p67phox and two membranes bound subunits, gp91phox and p22phox. During its activation cytosolic subunits moves to membrane subunit and forms the active NADPH enzyme complex (Bokoch and Knaus, 2003). Different reports have demonstrated the activation of NADPH oxidase causing dopaminergic neurodegeneration in vitro and in vivo (Gao et al., 2003; Wu et al., 2003). Here, we demonstrated that MPTP induced activation of microglial gp91phox in SN and orally administered mito-apocynin inhibited the NADPH oxidase activation (Fig. 9). These results clearly suggest that mito-apocynin inhibited MPTP-induced activation of NADPH oxidase and oxidative damages, thereby resulting in neuroprotection in the MPTP model.

Reactive glial cells mediated NO and NADPH oxidase mediated (O2.) reacts together and forms peroxynitrite (ONOO-), which induces oxidative stress to proteins by doing selective nitration to the tyrosine residues of dopaminergic neurons (Ara et al., 1998; Dawson and Dawson, 2003). In SN regions of PD patients, presence of activated glial cells that express iNOS and increased levels of nitrotyrosine are reported (Hunot et al., 1996). Previous studies have shown that in MPTP mouse model of PD, iNOS facilitates dopaminergic neurodegeneration via MPTP induced production of RNS and increase in nitrotyrosine levels (Liberatore et al., 1999; Dehmer et al., 2000). In our study, we found increased expression of 3-NT in dopaminergic neurons in MPTP-treated mice, but oral administration of mito-apocynin decreased the levels of TH nitrotyrosine in the SN of MPTP-treated mice (Fig. 10).
Consistent with peroxynitrite, 4-HNE is also a well-known marker for oxidative insult mainly caused by hydroxyl radical (·OH). The 4-HNE is an unsaturated aldehyde which is generated during lipid peroxidation in SN of PD brain compared to control (Yoritaka et al., 1996). Previous studies also demonstrated that 4-HNE blocked mitochondrial respiration and induced caspase dependent apoptosis in neurons (Picklo et al., 1999). We showed that a significant increase in 4-HNE expression 3 days after MPTP administration (Fig. 11). However, mito-apocynin significantly decreased the amount of 4-HNE in the SN of MPTP treated mice. Collectively, these results suggest that mito-apocynin decreases MPTP-induced oxidative stress, leading to dopaminergic neuronal survival.

The loss of dopamine and its metabolites is the most prominent biochemical change observed in PD patients and MPTP mouse model of PD (Jackson-Lewis and Przedborski, 2007). In our study, we also observed significant reduction of striatal neurotransmitters as well as behavioral imparities. However, mito-apocynin was able to restore the neurotransmitter levels and behavioral imparities in MPTP-treated mice nearly to the control level (Fig. 4 & 5). Consistently, mito-apocynin was found to protect the dopaminergic neurons and its terminals in nigrostriatum of MPTP-injected mice (Fig. 5). Neuroprotection afforded by mito-apocynin could be attributed either to partial restoration or prevention of further decreases in dopaminergic neurons in the nigrostriatum.

In conclusion, we have demonstrated that mito-apocynin improves behavioral imparities and protects nigrostriatal axis from MPTP toxicity by attenuating nigral activation of microglial and astroglial cells, reducing iNOS and pro-inflammatory cytokines, inhibiting the formation of active NADPH oxidase enzyme and decreasing oxidative stress in MPTP-
treated mice. Together, we propose that mito-apocynin may have therapeutic value in the treatment of PD and other disorders associated with inflammation and oxidative stress.

**References:**


Figure 1. Mito-apocynin protects dopaminergic neurons against MPP⁺-induced toxicity in primary neuronal culture. Mesencephalic tissues from E15 mouse embryos were cultured and grown on laminin-coated coverslips. The neuronal cultures were treated with 10μM MPP⁺ for 24 h in the presence or absence of 10μM mito-apocynin. After treatment, primary neurons were fixed with 4% paraformaldehyde and incubated with anti-TH antibody and viewed under a NIKON TE2000 fluorescence microscope. A. Tyrosine hydroxylase (TH) immunolabeling in primary mesencephalic culture from substantia nigra. B. TH-positive dopaminergic neuronal process length were quantified using MetaMorph image analysis software, as mentioned in Materials and Methods. C. Quantification of TH-positive dopaminergic neurons using MetaMorph software, as mentioned in Materials and Methods. Data represent the mean ± SEM of three to four experiments. ***, p < 0.001 vs. Control; @, p < 0.05 vs. MPP⁺; **, p < 0.01 vs MPP⁺.
Figure 2

A

Control

TH

MPI+ (M)

TH

p65

Hoechst

Merge

B

Control

MPP+ (M)

TH

GFAP

p65

Hoechst

Merge
**Figure 2. Effects of mito-apocynin on the translocation of NF-κB -p65 into nucleus of primary culture.** Mesencephalic tissues from E15 mouse embryos and day 1 old pups were cultured and grown on laminin-coated coverslips separately. The neuronal cultures from E15 mouse embryos were treated with 10μM MPP⁺ for 24 h in the presence or absence of 10μM mito-apocynin. The glial cultures from day 1 old pups were also treated with 10μM MPP⁺ for 24 h in the presence or absence of 10μM mito-apocynin. After treatment, cells were fixed with 4% paraformaldehyde and incubated with different antibodies and viewed under a NIKON TE2000 fluorescence microscope. **A.** Double labeling of TH and p65 in primary mesencephalic culture from substantia nigra (60X). *Inset,* higher magnified picture shows translocation of p65 into nucleus in MPP⁺-treated dopaminerculture. White arrowheads show expression of the markers. **B.** Double labeling of GFAP and p65 in primary glial culture from substantia nigra (60X). *Inset,* higher magnified picture shows translocation of p65 into nucleus in MPP⁺-treated astrocytes. White arrowheads show expression of the markers.
Figure 3. Mito-apocynin inhibits LPS-induced inflammatory processes in primary microglial cells. Primary microglial cells were isolated from C57BL/6 one day old pup and seeded in equal numbers on poly-d-lysine coated plates. Cells were treated with LPS (100ng/ml) in presence or absence of different doses of mito-apocynin (20µM or 40µM). A-C. After treatment, supernatants were collected and TNF-α, IL-6 and IL-12 proinflammatory cytokine levels were quantified using the luminex immunoassay system. D. Primary microglial cells from C57BL/6 one day old pups were treated with LPS (100ng/ml) in presence or absence of 20µM or 40µM of mito-apocynin. After 24 h of treatment, nitric oxide production was determined indirectly by quantifying the nitrite levels in the supernatant using the Griess reagent and a sodium nitrite standard curve. E. Primary
microglia from day 1 old pups of C57 BL/6 mice were treated with LPS (100 ng/ml) in presence or absence of miti-apocynin for 12 h and intracellular ROS generation was quantified using the fluorescent DCFDA dye. Data represent the mean ± SEM of three to four experiments. ***, p < 0.001 vs. Control; @, p < 0.01 vs. LPS; #, p < 0.001 vs LPS.
Figure 4. Mito-aocynin blocked MPTP-induced behavioral and locomotor deficits. Mice were administered mito-apocynin (3mg/kg/day) by oral gavage 24 h before MPTP treatment and continued for 5 days of co-treatment with MPTP (25mg/kg/day) and 4 days of post-
treatment with MPTP (25mg/kg/day). Control mice received 10% ethanol in saline. Mice were tested for motor activities 4 days after the last dose of MPTP. A. VersaPlot movement track of mice collected over 10 minutes was plotted. VersaMax data showing B. horizontal activity; C. vertical activity; D. distance travelled (cm.); E. movement time (sec.); F rest time (sec.); G. no. of stereotypy ; H. no. of rearing and I. time spent on rotarod (second) at 20 rpm rod speed. Data represent the mean ± SEM of six to eight mice per group. ***, p < 0.001 vs. the control group; **, p < 0.01 vs. the control group; *, p < 0.05 vs. the MPTP group; @, p < 0.001 vs. the MPTP group; #, p < 0.01 vs. the MPTP group.
Figure 5. Mito-apocynin prevents MPTP-induced neurotoxicity in the nigrostriatum of the mouse brain. Mice were administered mito-apocynin (3mg/kg/day) by oral gavage 24 h before MPTP treatment and continued for 5 days of co-treatment with MPTP (25mg/kg/day)
and 6 days of post-treatment of MPTP (25mg/kg/day). Control mice received 10% ethanol in saline. Seven days after the last MPTP injection, mice were sacrificed and Tyrosine hydroxylase (TH)-DAB immunostaining in striatum (A) and substantia nigra regions (B, upper panel 2X magnification; lower panel 10X magnification) were performed. 7 days after the last MPTP treatment, striatal (C) dopamine, (D) DOPAC and (E) HVA were measured by HPLC. Data represent the mean ± SEM of six to eight mice per group. ***, p < 0.001 vs. control; @, p < 0.01 vs. MPTP; #, p < 0.05 vs MPTP.
Figure 6. Mito-apocynin blocks activation of microglia and astrocytes in substantia nigra (SN) of MPTP treated mice. Mice receiving PBS as a control, MPTP (25mg/kg/day) or MPTP and mito-apocynin (3mg/kg/day) were sacrificed 3 days after the last dose of MPTP administration. Mouse SN tissues were immunostained with antibodies against IBA-
1 (marker of microglia) (A) and GFAP (marker of astrocytes) (B). Insets, four fold higher magnification of A-B. Dotted lines indicate the SNpc. Representative western blots illustrating the expression of IBA-1 (C) and GFAP (E) in SN. D. Bar graph showing means Western blot IBA-1/β-actin ratios ± SEM in SN of 6 mice per group. F. Bar graph showing mean Western blot GFAP/β-actin ratios ± SEM in SN of 6 mice per group. ***, p < 0.001 vs. the control group; *, p < 0.05 vs. the MPTP group.
Figure 7

A. Western blot analysis showing iNOS and β-actin expression in control, MPTP (M), and M + Mito-apo conditions.

B. Graph depicting the quantification of iNOS expression levels in Control, MPTP (M), and M + Mito-apo groups.

C. Immunofluorescence images showing the expression of IBA-1, iNOS, Hoechst, and Merge in Control, MPTP (M), and M + Mito-apo conditions.

D. Immunofluorescence images showing the expression of GFAP, iNOS, Hoechst, and Merge in Control, MPTP (M), and M + Mito-apo conditions.
Figure 7. Mito-apocynin decreases MPTP-induced nitric oxide damage in substantia nigra (SN) of MPTP treated mice. Mice were administered mito-apocynin (3mg/kg/day) by oral gavage 24 h before MPTP treatment and continued for 4 days of co-treatment with MPTP at a dose of 25mg/kg/day. Control mice received 10% ethanol in saline. Twenty-four h after the last dose of MPTP, mice were sacrificed. A. SN tissue lysates were analyzed by western blotting using antibody against iNOS. B. Bar graph showing means Western blot iNOS/β-actin ratios ± SEM in SN of 6 mice per group. C. Double labeling of GFAP and iNOS and (D) IBA-1 and iNOS in SN region of ventral midbrain. Images were captured at 20X and 60X (insets) magnification. Dotted lines indicate the SNpc. ***, p < 0.001 vs. the control group; *, p < 0.05 vs. the MPTP group.
Figure 8

Figure 8. Mito-apocynin inhibits the expression of iNOS and proinflammatory cytokines in ventral midbrain of MPTP-treated mice. Mice receiving PBS as a control, MPTP (25mg/kg/day) or MPTP and mito-apocynin (3mg/kg/day) were sacrificed 3 days after the last dose of MPTP administration. The mRNA expression of iNOS (A), TNF-α (B), IL-1β (C) and IL-6 (D) was analyzed by quantitative real-time PCR. Data represent the mean ± SEM of six mice per group. **, p < 0.01 vs. control; *, p < 0.05 vs. control; @, p < 0.05 vs MPTP.
Figure 9. Mito-apocynin attenuates activation of NADPH oxidase in SN of MPTP-treated mice. Mice were administered mito-apocynin (3mg/kg/day) by oral gavage 24 h prior MPTP treatment and continued for 4 days of co-treatment with 25mg/kg/day MPTP. Control mice received 10% ethanol in saline. Twenty-four h after the last dose of MPTP, mice were sacrificed. A. Representative western blots illustrating the expression of...
gp91phox (membrane bound subunit of NADPH oxidase) in SN.  

**B.** Bar graph showing means Western blot gp91phox/β-actin ratios ± SEM in SN of 6 mice per group.  

**C.** Substantia nigra tissue sections were double labeled for gp91phox and IBA-1. Images were captured at 20X and 60X (insets) magnification. The SNpc zone is outlined in white dots. *Inset* pictures demonstrated colocalization of IBA-1 and gp91phox. **, p < 0.01 vs. the control group; *, p < 0.05 vs. the MPTP group.
Figure 10. Mito-apocynin decreases the formation of 3-nitrotyrosine (3-NT) in SN of MPTP treated mice. Mice were administered mito-apocynin (3mg/kg/day) by oral gavage 24 h before MPTP treatment and continued for 4 days of co-treatment with 25mg/kg/day MPTP via i.p. Control mice received 10% ethanol in saline. Twenty-four h after the last dose
of MPTP, mice were sacrificed.  **A.** Representative western blots illustrating the expression of 3-NT in SN.  **B.** Bar graph showing means Western blot 3-NT/β-actin ratios ± SEM in SN of 6 mice per group.  **C.** Double labeling of TH (marker of dopaminergic neurons) and 3-NT in SN region of ventral midbrain. Images were captured at 20X and 60X (insets) magnifications. The SNpc zone is outlined in white dots. Inset pictures demonstrated colocalization of TH and 3-NT. ***, p < 0.001 vs. the control group; *, p < 0.05 vs. the MPTP group.
Figure 11. Mito-apocynin inhibits MPTP induced formation of 4-hydroxynonenol (4-HNE) in SN in vivo. Mice were administered mito-apocynin (3mg/kg/day) by oral gavage 24 h before MPTP treatment and continued for 4 days of co-treatment with 25mg/kg/day MPTP via i.p. Control mice received 10% ethanol in saline. Twenty-four h after the last dose
of MPTP, mice were sacrificed. **A.** Representative western blots illustrating the expression of 4-HNE in SN. **B.** Bar graph showing means Western blot 4-HNE/β-actin ratios ± SEM in SN of 6 mice per group. **C.** Double labeling of TH (marker of dopaminergic neurons) and 4-HNE in SN region of ventral midbrain. Images were captured at 20X and 60X (insets) magnifications. The SNpc zone is outlined in white dots. Inset pictures demonstrated colocalization of TH and 4-HNE. ****, p < 0.01 vs. the control group; ***, p < 0.001 vs. the MPTP group.
CHAPTER VI
GENERAL CONCLUSIONS

This section presents a general overview of the results and findings described in this dissertation, with a special emphasis on the future directions and overall implications of these findings on the pathogenesis of Parkinson’s disease. The major findings pertaining to each research chapter and their specific implications are covered in the discussion section of the relevant chapters.

Pin1, a peptidyl-prolyl isomerase, is differentially activated in neuronal and glial cells of the substantia nigra

Reversible phosphorylation on Ser/Thr – Pro (S/T-P) motifs regulated by Pro-directed protein kinases and phosphatases is an important molecular switch in controlling various cellular processes (Gothel and Marahiel, 1999). Since the discovery of protein interacting with never in mitosis – A (Pin1), a lot has become known about the conformational importance of Pro-directed kinases in cellular signaling (Uchida et al., 1999). Pin1 is unique among the peptidyl-prolyl isomerases in that it specifically recognizes phosphorylated serine or threonine residues immediately preceding proline (pSer/Thr-Pro) in a subset of proteins and isomerizes the cis/trans conformation of peptide bond (Joseph et al., 2003; Lu, 2004). Recently, Ryo et al., reported that Pin1 overexpression facilitates formation of α-synuclein inclusions in a cellular model of α-synuclein aggregation and Pin1 also localizes in Lewy bodies in PD patients (Ryo et al., 2006). But the level, activity and the role of Pin1 in the inflammatory responses in the pathogenesis of PD are completely unknown. Results from chapter II and chapter III clearly demonstrate that dopaminergic neurons and glial cells (both
microglia and astrocytes) express Pin1 in the cytosol, and the level of Pin1 upregulates during disease states. Deregulation of Pin1 activity has been proposed in different diseases. We identified that Pin1 is highly expressed in the pigmented dopaminergic neurons of PD patients in comparison to age-matched controls. Human PD cases are associated with loss of pigmented dopaminergic neurons in SN, and our findings of elevated Pin1 expression only in pigmented dopaminergic neurons suggest a possible role of Pin1 in the pathogenesis of PD. We show in this study for the first time that Pin1 may also be a possible therapeutic target for PD. Additionally, Pin1 levels increases significantly in response to different toxic stimuli in cell culture and animal models of PD. Recent findings have demonstrated that Pin1 inhibition has anti-cancer effects in experimental studies (Dourlen et al., 2007). Among various Pin1 inhibitors, Juglone (5-hydroxy-1,4-napthalenedione, C_{10}H_{6}O_{3}) has been widely used in research fields. It is an aromatic organic napthoquinone, naturally found in the leaves, roots and bark of black walnut plants (Esnault et al., 2008; Jeong et al., 2009). In our experiment, Juglone not only inhibits Pin1 expression, but also shows anti-inflammatory and neuroprotective effects in cell and animal models of PD, suggesting the potential of Pin1 as a therapeutic target in the progression of PD.

The precise signaling mechanism and downstream targets of Pin1 during dopaminergic neurodegeneration are not known yet. However, potential downstream targets for regulation by Pin1 can be inferred based on the known substrates and functions of Pin1 in cancerous and innate immune cells. Previous reports indicated Pin1’s role in controlling several transcription factors that mediate induction of iNOS, a key inflammatory molecule involved in the pathogenesis of PD (Gothel and Marahiel, 1999; Shen et al., 2005). In cancerous cells, Pin1 has been shown to bind to the phosphorylated Thr254-Pro residue of
transcription factor NF-κB p65. Through this interaction, Pin1 helps stabilize p65 in the nucleus, maintaining activation of NF-κB (Ryo et al., 2003). NF-κB activation is a key inflammatory mediator and Ghosh et al. (2007) previously reported induction of NF-κB in SN of PD patients and MPTP-treated mice. In our study, different types of stimulus mediated activation of Pin1 in glial cells and its association with p65 strongly suggest that Pin1 controls the NF-κB-mediated inflammatory processes in PD. Additionally, depletion of Pin1 by small interfering RNA reduces oxidative stress in microglial cells. Overall, this study demonstrates that Pin1 is a key upstream signaling molecule of inflammation and oxidative stress, and inhibition of Pin1 activation rescues the dopaminergic neurons from neurotoxicity.

**Antinflammatory and neuroprotective properties of novel compounds diapocynin and mito-apocynin**

The findings in chapter IV and V demonstrate that by attenuating inflammation and oxidative stress, the novel compounds diapocynin and mitoapocynin protect dopaminergic neurons in nigrostriatum. Parkinson’s disease is a multifactorial disease, with glial activation,
inflammation, oxidative stress and mitochondrial dysfunction playing a central role in dopaminergic neurodegeneration, specifically in the nigrostriatum. For the last few years, there have been increased efforts to search for neuroprotective agents to protect against the irreversible loss of neurons. Administration of a dopamine agonist or levodopa has been the widely used treatment for PD symptoms, but this treatment does not affect the disease pathogenesis. Dopaminergic neuroprotection in animal models of PD has been demonstrated with various substances, including glial cell line derived neurotrophic factor (GDNF), brain derived neurotrophic factor (BDNF) and TGF-β (Kordower et al., 2000). Additionally, various anti-inflammatory agents such as NSAIDs, COX inhibitors, statins, pioglitazone, minocycline have been used in different animal models of PD (Chen et al., 2003; Ghosh et al., 2009). However, most of these compounds failed in either preclinical trials or in human phase I trials due to their inability to cross the blood-brain barrier or due to limited bioavailability. Moreover, these agents also cause side effects and toxicity. Hence, understanding the mechanism of the disease process and development of a successful neuroprotective therapeutic approach to halt the disease progression are of principal importance in the PD research field. MPTP causes glial activation and inflammation within 24 h of its administration and initiates a self-perpetuating dopaminergic neurodegenerative process beginning at 72 h (Ghosh et al., 2009). Here, we clearly demonstrate that diapocynin (a metabolite of apocynin) crosses the blood brain barrier, attenuates nigral activation of microglial and astroglial cells, and inhibits proinflammatory molecule (iNOS) and production of NADPH oxidase mediated superoxide formation. Additionally, it also decreases oxidative stress, improves behavioral functions and protects the nigrostriatum in MPTP-treated mice.
These results suggest its potential as a therapeutic candidate in clinical trials of human PD patients.

Mitochondrial dysfunction due to oxidative stress, mitochondrial DNA deletions, altered mitochondrial morphology, and interaction of pathogenic proteins with mitochondria results in dopaminergic neurodegeneration. Consequently, therapeutic approaches targeting mitochondrial dysfunction and related oxidative stress have great promise in the cure of PD. MPTP and other complex-I inhibitors such as rotenone, maneb, paraquat, fenzaquin and trichloroethylene result in loss of nigral dopaminergic neurons in a mouse model of PD, implicating mitochondrial dysfunction in the disease pathogenesis (Betarbet et al., 2000; Gash et al., 2008).

Moreover, reduced complex-I activity and an increased susceptibility to MPP⁺, the toxic metabolite of MPTP, were also observed in mitochondrial DNA from PD patients, clearly demonstrating the mtDNA encoded defects in PD (Swerdlow et al., 1996; Gu et al., 1998). Based on these observations, intervening one of these processes should alleviate harmful effects of mitochondrial dysfunction. Numerous bioenergetics agents that
improve mitochondrial function, including creatine and CoQ_{10} are in clinical trial for PD. Recently, we (Ghosh et al., 2010) demonstrated that mitochondria targeted antioxidant Mito-Q_{10}, which is a chemical modification of Co-Q, could protect dopaminergic neurons from MPTP-induced toxicity in mice (Ghosh et al., 2010). Similarly, we synthesized a novel mitochondria targeted anti-oxidant, mito-apocynin, which is also a derivative of apocynin and tested its efficacy in cell culture and animal models of PD. Herein, we demonstrate that mito-apocynin protects against MPP^{+}-induced loss of dopaminergic neurons in primary mesencephalic culture by reducing glial cell mediated inflammatory reactions. Moreover, administration of mito-apocynin in mice protects the dopaminergic neurons and terminals from MPTP toxicity by reducing inflammatory reactions and oxidative stress. Collectively, these results strongly suggest that additional preclinical development of mito-apocynin may yield an effective neuroprotective drug for treatment of PD.
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ACKNOWLEDGEMENT

I would like to sincerely and profoundly thank my research advisor Dr. Anumantha G. Kanthasamy, for allowing me to join his lab and for supporting me entirely during the graduate study program. During my Ph.D. training at his lab, I have acquired and developed numerous skills in designing good scientific projects, interpret scientific data, pertinent questions in progressing the projects, critical independent thinking and writing etc. His broad knowledge in the field of neurodegenerative disorders and constructive advises, encouragement and support have been a great value to my accomplishments during graduate studies and research. Without his relevant suggestions and guidance, it would have been impossible to come up with this Ph.D. thesis.

I would also like to extend my deep sense of gratitude to my committee members, Dr. Arthi Kanthasamy, Dr. Marian L. Kohut, Dr. Ann Smiley-Oyen and Dr. N. Matthew Ellinwood for their constant advice and constructive criticism which went a long way in improving the quality of my research. I would like to sincerely thank Dr. Vellareddy Anantharam for interventions in getting the resources required for the study, critically evaluating manuscripts and being supportive throughout my graduate work. I would also like to thank Dr. Balaraman Kalyanaraman at the Medical College of Wisconsin, Milwaukee for generously providing us the diapocynin and mito-apocynin compounds.

I also owe a big debt of thanks to all my lab mates, past and present without whom the bench research would have been incredibly harder. They are Dr. Hariharan Swaminathan, Colleen Jeffrey, Dilshan Harischandra, Muhammet Aye, Dr. Pallavi Srivastava, Dr. Huajun
Jin, Dr. Chunjuan Song, Dr. Richard Gordon, Dr. Arunkumar Asaithambi, Dr. Afeseh Ngwa Hilary, Daquing Huang, Dongsuk Kim, Dustin Martin, Matthew Neal, Nikhil Panicker, Dr. Prashant Chandramani, Sreemoyee Acarya, Grace huh, Sri Harsha Kanuri, Monica Langley and Dan Luo. I also want to profoundly thank Maryann Devries for her kind assistance in arranging relaxing events and planning fun.

I want to also gratefully acknowledge the support from past and current Biomedical Sciences Staff, Kim M. Adams, William B. Robertson, Linda Erickson, Cathy Martens, and Nada Paavlovic.

Finally, I would like to express gratitude to my loved ones - my wife - Poulomi, my parents, my family and my friends for their ample and unconditional support, without which it would have been near impossible to finish this task. I would like to dedicate the dissertation to all my family members.