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Characterization of the MitoPark mouse model of Parkinson's disease for neurotoxicity and neuroprotection studies

Monica Langley
Iowa State University

Characterization of the MitoPark mouse model of Parkinson's disease for neurotoxicity and neuroprotection studies

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Monica Langley

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Program of Study Committee:
Anumantha G. Kanthasamy, Major Professor
Arthi Kanthasamy
Wilson Rumbeiha
Manju Reddy
Jason Ross

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ABSTRACT

Parkinson's disease (PD) is a chronic, progressive neurodegenerative disorder affecting about one million people in the United States and an estimated 10 million people worldwide. Although the pathological features of the disease, including dopaminergic neuronal loss and protein aggregation in the nigrostriatal tract are well understood, no cure or therapy exists that can decelerate or halt the neurodegenerative process. The exact mechanisms leading to cell death are still unresolved and advances in drug discovery for PD have been hampered by the lack of animal models that faithfully recapitulate the chronic, progressive nature of the disease, the full range of symptomology, and the underlying pathophysiological pathways. A recent transgenic mouse model, referred to as "MitoPark", was created by selective inactivation of mitochondrial transcription factor A (TFAM) in the nigrostriatal pathway through control of the dopamine transporter (DAT) promotor by utilizing Cre/LoxP recombination. MitoPark mice model human PD by exhibiting a progressive course of the phenotypic manifestations and neurodegeneration, protein inclusions in nigral tissues, L-DOPA responsiveness, and adult-onset of disease. Considering that nonmotor symptoms reduce the quality of life and increase the cost of healthcare for PD patients, we characterized the full range of symptomatology in the MitoPark model. Similar to human PD, our MitoPark data suggest that many nonmotor symptoms, including cognitive deficits in learning and memory, olfactory discrimination, and neuropsychiatric deficits, are present in the model prior to severe motor dysfunction. Moreover, we have identified changes in neurogenesis, oxidative stress markers, and neurochemistry in the brain that correlate with the nonmotor symptoms observed. Furthermore, we have established that

neuroinflammation (as a result of reactive microgliosis) and altered neurogenesis (subsequent to dopamine depletion) are present in later stages of the disease in this mouse model, making it particularly valuable for translational neurotoxicity and drug discovery studies. We also demonstrate that manganese, an environmentally relevant toxin linked to increased PD risk, can exacerbate some of these effects and accelerate disease onset in MitoPark mice. Finally, we explore two promising therapeutic options for PD: a mitochondrially targeted antioxidant and a neuroprotective protein. Our data demonstrate that Mito-apocynin treatment effectively attenuates progressive motor deficits, neuroinflammation, oxidative stress, and neurodegeneration in a comprehensive PD model via NOX2 inhibition in microglia and antioxidant effects in neurons. By expanding on recent work in our laboratory showing that Prokineticin-2 (PK2) serves a compensatory neuroprotective role in PD models, we identified a new mechanism by which PK2 may exert its effect: promoting neural stem cell proliferation and differentiation. Taken together, we have identified salient features of PD disease progression in a mouse model and applied the model to elucidate gene-environment interactions and to evaluate novel therapeutic strategies.

CHAPTER 1

GENERAL INTRODUCTION

Dissertation Organization

The alternative format was chosen for this dissertation and consists of manuscripts that have either been published or are in preparation for submission. There is a general introduction containing two literature reviews, four research chapters in the format of their corresponding journals, and a general conclusions/future directions section. Each research chapter contains references in the format of the journal it was or will be submitted to. Introduction, literature reviews, and conclusion sections have the references combined at the end of the thesis. Chapter 1 gives a brief overview of Parkinson's disease, environmental factors contributing to the disease, and the animal models currently in use. The Background and Literature Review-I section provides and in-depth review of current literature regarding nonmotor symptoms of Parkinson's disease. The Background and Literature Review-II discusses novel therapeutic strategies being developed for the treatment of Parkinson's disease.

Chapter 2 is a research chapter exploring the nonmotor symptoms of Parkinson's disease in a novel MitoPark transgenic mouse model. This Chapter will be submitted to the Journal Brain and is formatted as such. Chapter 3 is a manuscript that is currently under revision for publication in Neurotoxicology. It reveals that manganese can exacerbate and accelerate Parkinson's like disease progression and motor deficits in the MitoPark model. Chapter 4 is from a recently accepted publication in Antioxidants and Redox Signaling that

identified the neuroprotective efficacy of a novel mitochondrially targeted antioxidant compound in MitoPark mice. Chapter 5 studies the role of a secreted peptide, Prokineticin-2, in altered neurogenesis and nonmotor symptoms in the MitoPark model and is formatted for submitting to Scientific Reports.

For this dissertation, the experimental data and results were obtained by the author during the course of her PhD study in the Interdepartmental Toxicology Graduate Program under the supervision of her major professor, Dr. Anumantha G. Kanthasamy at Iowa State University, Department of Biomedical Sciences.

Introduction

Parkinson's disease

Parkinson's disease (PD) is a debilitating neurodegenerative disorder, estimated to affect 10 million people worldwide, and increasing by 60,000 new cases annually (Dauer and Przedborski, 2003). Age is a risk factor for PD, with about 3% of those over 60 years being affected by the disorder and almost 5% being affected in those over 85 years old (Mizuno et al., 2001). Early onset PD has been linked with various mutations in genes including α-synuclein, Parkin, leucine rich repeat kinase 2 (LRRK2), PINK1, and DJ-1 (Bonifati et al., 2008; Gwinn-Hardy, 2002; Tang et al., 2006; Weng et al., 2007). Pathological features of the disease include a loss of dopaminergic neurons in the substantia nigra (SN), loss of dopamine in the axon terminals in the striatum, and accumulation of protein aggregates containing alpha-synuclein in the form of Lewy bodies and Lewy neurites (Spillantini et al., 1998). Because these pathologies are difficult if not impossible to detect in living patients, diagnosis is typically done based off of clinical signs including

motor symptoms of such as bradykinesia, tremor, rigidity, and postural instability. A number of nonmotor symptoms also impair quality of life for PD patients and contribute to healthcare costs. The exact etiology of the idiopathic disease still remains unknown, but it is thought that genetic and environmental factors converge on cell death mechanisms underlying the nigrostriatal neurodegeneration (Pissadaki and Bolam, 2013). Mitochondrial dysfunction, oxidative stress, and neuroinflammation are all thought to contribute to pathophysiological processes in PD (Subramaniam and Chesselet, 2013; Varçin et al., 2012).

Environmental risk factors

More than 90% of PD cases are not caused by the known genetic mutations and are instead considered sporadic form. Hence, a 'multi-hit' hypothesis has been proposed which assumes that multiple neurotoxic insults and other causative factors accumulate over an individual's lifetime and culminate on the development of the neurodegenerative disorder (Carvey et al., 2006). A number of environmental factors increase risk for the development of PD including pesticide exposure, rural living, agricultural occupation, well water drinking, prior

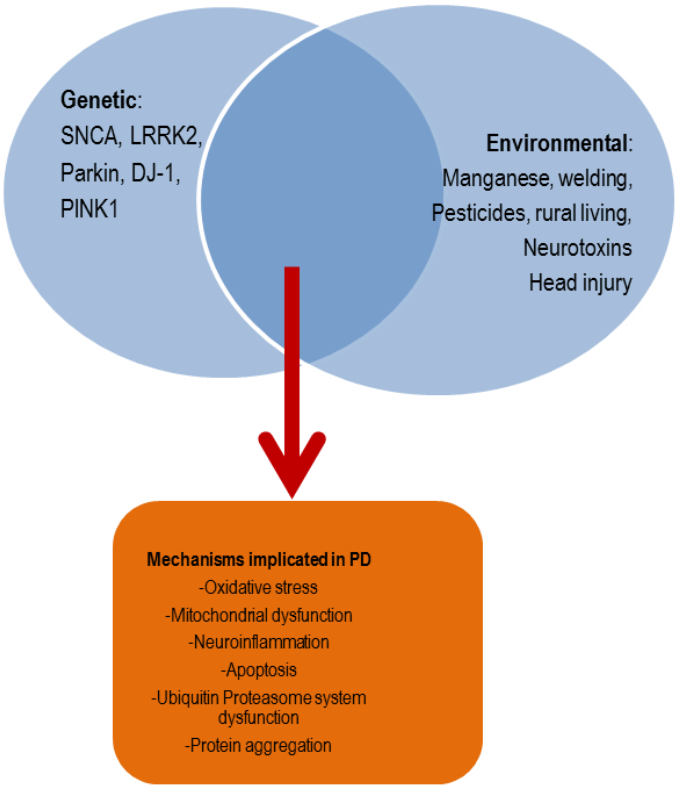


Figure 1. *Etiology of Parkinson's disease involves genetic and environmental influences*

head injury, age, and beta-blocker use (Kalia and Lang, 2015). Increased and decreases odds ratios based on certain genetic polymorphisms or epigenetic changes have also been associated with developing PD. Decreased risk is associated with tobacco use, coffee drinking, NSAID use, calcium channel blocker use and alcohol consumptions (Kalia and Lang, 2015).

Manganese

Manganese exposure has also been implicated as a risk factor for various neurological disorders, including PD (Haynes et al., 2015; Lucchini et al., 2014; Sanders et al., 2015; Sikk and Taba, 2015). Manganese neurotoxicity is a concern in occupational settings, especially for those working in ceramics, welding, steel making and mining industries (Mielke et al., 2002; Yabuuchi and Komaba, 2014). Excessive exposure to manganese or failure to excrete it can cause accumulation of manganese in the basal ganglia leading to manganism, a movement disorder similar to PD (Bouabid et al., 2015; Peres et al., 2016b). Symptoms of manganism mimic PD symptoms, including motor deficits such as rigidity and bradykinesia, but manganism patients present a characteristic "cock-walk" gait and neuropsychiatric symptoms often occur earlier than later (Bowler et al., 2006; Bowler et al., 1999; Kwakye et al., 2015). Unlike PD, manganism affects the neurons located in the globus pallidus. (Criswell et al., 2015; Perl and Olanow, 2007). Since patients with manganism respond poorly to levodopa therapy, chelation therapy has been used as an alternative (Discalzi et al., 2000; Ky et al., 1992).

Recent studies suggest that manganese and other transition metals may have an etiological role in PD and may accelerate the progression of PD; a recent study has shown a

dose-dependent increase in UPDRS3 scores in welders (Gorell et al., 1999; Racette et al., 2016). Welders exposed to manganese fumes display atypical neurobehavioral changes that correlate with accumulation of manganese in the basal ganglia when measured via MRI (Lee et al., 2016a; Lewis et al., 2016). Genetic defects in manganese transporter proteins can cause metal-induced Parkinsonism, including a form of childhood-onset Parkinsonism caused by an autosomal recessive manganese transporter defect leading to accumulation of manganese. Chronic exposure to heavy metals and pesticides has also been connected with earlier onset of sporadic PD (Ratner et al., 2014). Mutations in a manganese specific efflux transporter (SLC30A10), believed to protect cells from Manganese induced toxicity, can cause familial Parkinsonism (Chen et al., 2015a; Leyva-Illades et al., 2014).

While the exact pathogenic mechanisms of manganese-induced neurotoxicity are not well understood, evidence suggests manganese promotes protein aggregation, much like classical Parkinsonian toxins. Manganese can also inhibit mitochondrial complexes I and II of the electron transport chain leading to oxidative stress. (Aschner et al., 2009; Carboni and Lingor, 2015; Liu et al., 2013; Peres et al., 2016a; Zheng et al., 1998). Additionally, manganese is capable of directly affecting neurons by triggering glial activation and neuroinflammation via microglia and astrocytes (Filipov and Dodd, 2012; Moreno et al., 2011; Moreno et al., 2009; Streifel et al., 2012). While striatal dopamine loss is a hallmark of PD models, studies have shown conflicting results in manganese treated rodent studies (Filipov and Dodd, 2012; Moreno et al., 2011; Moreno et al., 2009; Streifel et al., 2012).

Interactions between environmental and genetic factors play a major role in the pathogenesis of PD. Manganese and mutations associated with PD can alter biochemical pathways which can progress the development of PD (Bornhorst et al., 2014; Chen et al.,

2015; Roth, 2014), but little evidence exists on manganese exposure aggravating Parkinsonism in animal models. PD related genes are known to mediate manganese-induced toxicity in cell culture and animal models (Bornhorst et al., 2014; Higashi et al., 2004; Lovitt et al., 2010; Tan et al., 2011), unfortunately none of the existing toxin based and genetic models of PD fully recapitulate the progressive nature of the disease. Understanding how manganese promotes the pathophysiological processes involved in PD could help develop better strategies to prevent or delay PD onset and progression.

Animal models of PD

Toxin-based models

MPTP is a common toxin-based PD model in mouse and non-human primate studies. The drug was first discovered by neurologist William Langston when a group of people accidentally injected the drug and displayed features similar to PD (Tetrud et al., 1989). 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is converted to 1-methyl-4-phenylpyridinium (MPP^+) by glial cells. In dopaminergic neurons, MPP^+ inhibits mitochondrial Complex I (Blesa and Przedborski, 2014; Varastet et al., 1994). One advantage of this model is the strong affinity of MPP^+ for dopamine transporter over other monoamine transporters (Javitch et al., 1985). Both acute and chronic treatment paradigms are possible, each capturing key pathological features of PD. Acute MPTP treatment is typically used for study of neuroinflammation; whereas, chronic or subchronic studies are used in studies addressing neurodegeneration (Fornai et al., 2005; Ghosh et al., 2016). Although MPTP does not affect rats, it is a useful model in non-human primates (Kowall et al., 2000; Riachi et al., 1990). Its ability to cross the blood-brain-barrier makes administration more practical for

experimenters (Jagmag et al., 2015). A serious limitation in the MPTP model is the absence of Lewy Body-like protein inclusions (Blesa and Przedborski, 2014). Another caveat is that drugs may interact with the metabolic pathways of MPTP, confounding therapeutic effects by limiting MPP^+ concentration rather than true mitigation of neurodegeneration (Jagmag et al., 2015).

In rats, 6-hydroxydopamine (6-OHDA) is used to model PD (Thiele et al., 2012; Ungerstedt, 1968). 6-OHDA has to be injected into the substantia nigra or striatum because it cannot cross the blood-brain-barrier. Intranigral injection quickly results in a reproducible lesion, while striatal injection causes neurodegeneration in dopaminergic neurons and motor deficits over 1-3 wks **(**Jeon 1995; Sauer and Oertel 1994). Disadvantages of this model include unspecific targeting of both dopaminergic and noradrenergic transporters, extranigral pathology, and no Lewy Body-like inclusions (Dauer and Przedborski, 2003).

The pesticides rotenone and paraquat have also been used to model PD. Rotenone is a mitochondrial Complex I inhibitor, similar to MPP^+. Systemic rotenone studies performed in rats include intracellular inclusions and nigrostriatal dopaminergic neurodegeneration (Blesa and Przedborski, 2014; Jagmag et al., 2015; Sherer et al., 2003). Stereotaxic injection of rotenone results in high mortality (Fleming et al., 2004; Xiong et al., 2009). Paraquat treatment produces neuronal damage by generation of reactive oxygen species and results in Lewy Body Formation (Cicchetti et al., 2005; Miller, 2007; Thrash et al., 2007).

Lipopolysaccharide (LPS) is found in the cell wall of gram negative bacteria and causes strong inflammatory responses. Intranigral or intraperitoneal injection has been used

to model neuroinflammatory changes associated with PD (Hsieh et al., 2002; Panicker et al., 2015; Qin et al., 2007).

Genetic models

Genetic models can help to unfold signaling pathways and enable screening of novel therapeutics. One gene linked to dominant types of familial PD is the SNCA gene which encodes α-synuclein, a major constituent of Lewy bodies. Five mutations in α-synuclein have been located in familial PD cases (Appel-Cresswell et al., 2013; Chartier-Harlin et al., 2004; Kiely et al., 2013; Kruger et al., 1998; Proukakis et al., 2013; Singleton et al., 2003; Zarranz et al., 2004).

A number of transgenic α-synuclein models exist which overexpress wild type or mutant α-synuclein, though many do not recapitulate all features of PD: SN neuronal loss, inclusion bodies, and motor impairment (Dehay and Fernagut, 2016). Of these, the wild type/A53T overexpressing mice controlled under the CMVE-Syn 1 promotor best recapitulates PD pathology and symptomology (Dehay and Fernagut, 2016; Oliveras-Salva et al., 2013). Injection of α-synculein protein or virus into the rodent brain caused dopaminergic cell loss and inclusion bodies in various studies (Dehay and Fernagut, 2016; Engeln et al., 2013; Lauwers et al., 2007; Oliveras-Salva et al., 2013; Oueslati et al., 2012). Nonmotor symptoms olfactory, gastrointestinal, cognitive and autonomic dysfunction have been identified in various α-synuclein models but were not universally identified in any one particular model (Hallett et al., 2012; Jagmag et al., 2015; Taylor et al., 2009).

A late-onset, autosomal dominant familial form of PD is caused by gene mutations in LRRK2. Mutations in the LRRK2 gene are also a major risk factor contributing to idiopathic

Table 1. *Rodent models of PD. NA= not available; ND= not determined; +=present; -=absent.*

	Model	Progressive neurodegeneration	DA loss	L-DOPA responsive	Mitochondrial dysfunction	Lewy Body-like inclusions	Motor symptoms	Non- motor symptoms
genetic	**α-synuclein** (A53T)	+	-	+	+	+	+	+ olfactory,GI,cognitive; - anxiety; ND: sleep, depression
genetic	**LRRK2** (R1441G)	-	-	+	+	-	+	+GI -olfaction, mood, cognitiive; ND sleep
genetic	**Parkin** (exon 3 deletion)	-	+	NA	+	-	+	+/- anxiety, cognitive; -depression, olfaction; ND others
genetic	**Pink1**	-	-	NA	+	-	-	-anxiety; ND others
genetic	**DJ-1**	-	-	-	+	-	+	-anxiety; ND others
genetic	**MitoPark**	+	+	+	+	+	+	+ circadian rhythm +cognitive deficits ND others
toxin	**6-OHDA**	-	+	+	+	-	+	+depression; -anxiety, cognitive ND olfactory, GI
toxin	**MPTP**	-	+	+	+	-	+	+ olfactory,sleep,cognitive +/- GI; - anxiety, depression
toxin	**Rotenone**	+	+	NA	+	+	+	+gastrointesting +depression

PD. Transgenic LRRK2 lines have largely shown protein aggregation without nigrostriatal degeneration (Chen et al., 2012; Li et al., 2007). None of the current LRRK2 models produce dopaminergic neuronal loss, inclusion bodies, and motor impairment together. Most LRRK2 models only contained one of these PD traits at most (Ramonet et al., 2011; Xiong et al., 2017). Recently, olfactory and gastrointestinal dysfunction were identified in separate LRRK2 transgenic mouse models (Bichler et al., 2013; Dranka et al., 2014).

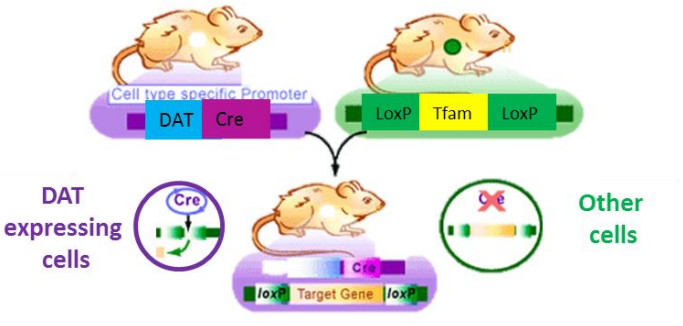
Recessive mutation or deletions in Parkin, PINK1, and DJ-1 genes cause early onset Parkinsonism. However, Parkin, DJ-1, and PINK1 transgenic mouse models have not shown evidence of inclusion bodies, and most do not show SN neuron loss (Rousseaux et al., 2012). Recent rat PINK1 and DJ-1 knockout models in rats seemed to resemble PD by exhibiting dopamine loss and motor impairment, but not protein aggregates (Dave et al., 2014; Jagmag et al., 2015). Moreover, they did not show evidence of nonmotor symptoms such as anxiety-like phenotype or have a progressive onset of motor deficits and neurodegeneration.

Many other genetic PD models exist in the fruit fly and *C. Elegans* (Bornhorst et al., 2014; Chen et al., 2015). These models have been utilized for identifying gene-environment interactions for better understanding of PD etiology. Unfortunately, none of the genetic models mimicking human gene mutations or deletions currently available faithfully replicate the disease progression and pathology as observed in clinical PD. This substantiates the idea of a multi-hit hypothesis in the underlying mechanisms contributing to the disease development.

Recently, models to mimic pathway perturbations relevant to PD progression have been established. For example, mice lacking Pitx3 have a loss of nigrostriatal neurons, recapitulating behavioral deficits related to dopaminergic depletion (Hwang et al., 2005). Mice with reduced monoamine storage capacity due to decreased VMAT2 expression showed both motor and nonmotor symptoms of PD including delayed gastric emptying, olfactory deficits, altered sleep latency, and neuropsychiatric effects (Taylor et al., 2009). Pitx3, VMAT, and DAT genes are all associated with increased PD risk (Antony et al., 2011; Hwang et al., 2005; Taylor et al., 2009).

MitoPark mouse model

A recently developed transgenic model of PD utilized Cre/LoxP recombination to selectively knockout the mitochondrial transcription factor TFAM in



(Adapted from http://www.scq.ubc.ca/targeting-your-dna-with-the-crelox-system/)

Figure 2. *Breeding schematic of MitoPark mice*

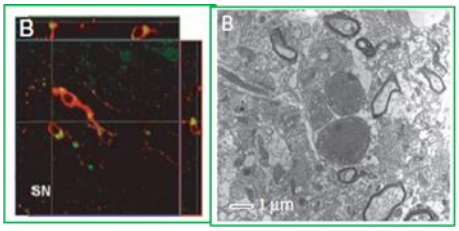


Figure 3. *Protein inclusions and abnormal mitochondria in MitoPark mice.Adapted from Ekstrand et al., 2007.*

cells expressing DAT (Ekstrand et al., 2007). This model, deemed "MitoPark" mice, have a chronic, progressive neurodegeneration accompanied by progressive motor deficits, intraneuronal inclusions, and dopamine loss. Importantly, Ekstrand et al crossed the mice with α-synuclein knockout mice to be certain of whether or not the observed protein inclusions were indeed Lewy bodies. In the α-synuclein knockout crosses, the aggregates persisted and were thought to instead consist of mitochondrial proteins. This model is also L-DOPA responsive and has recently been shown to involve nonmotor aspects of the disease as well (Fifel and Cooper, 2014; Gellhaar et al., 2015; Li et al., 2013b).

For example, spatial learning but not memory deficits were identified at 8 and 20 wks of age using the Barnes Maze and novel object recognition tests (Li et al., 2013). Psychosis-like side effects were observed upon treatment with DA agonists but not A2A antagonist (Grauer et al., 2014). Circadian rhythm dysfunction became apparent upon high intensity light-on conditions and at later ages, a loss of circadian rhythms was seen (Fifel et al., 2014).

Since the model was developed, more detailed pathological features have been identified by various groups. At 6-10wks, impaired electrophysiological parameters were found in dopaminergic neurons, and increased L-type calcium channel mRNA and PK2 protein expression were observed in MitoPark mice versus their littermate controls (Branch et al., 2016; Gordon et al., 2016). At 28-30wks, increased astrocyte marker GFAP, greater glutamate release in the striatum, and white matter MRI changes were identified (Farrand et

al., 2016). Also at 30 wks, MRI changes indicative of iron accumulation were found in the substantia nigra (Farrand et al., 2016).

Few neuroprotection studies have been performed to date in the MitoPark model. First and most importantly, it was shown that MitoPark mice are L-DOPA responsive and that dyskinesia develops over time (Gelhaar et al., 2015). LRRK2 kinase inhibition showed no improvement in this model (Fell et al., 2015). A positive allosteric modulator of mGluR4 helped only in conjunction with L-DOPA (Le Poul et al., 2012). PTEN KO dopaminergic neuron grafts had increased survival and protected against some motor deficits versus traditional grafted cells (Zhang et al, 2012). An A2A antagonist helped restore locomotor function (Marcellino 2010). However, no studies aimed at impeding pathophysiological pathways or addressing nonmotor symptoms of PD.

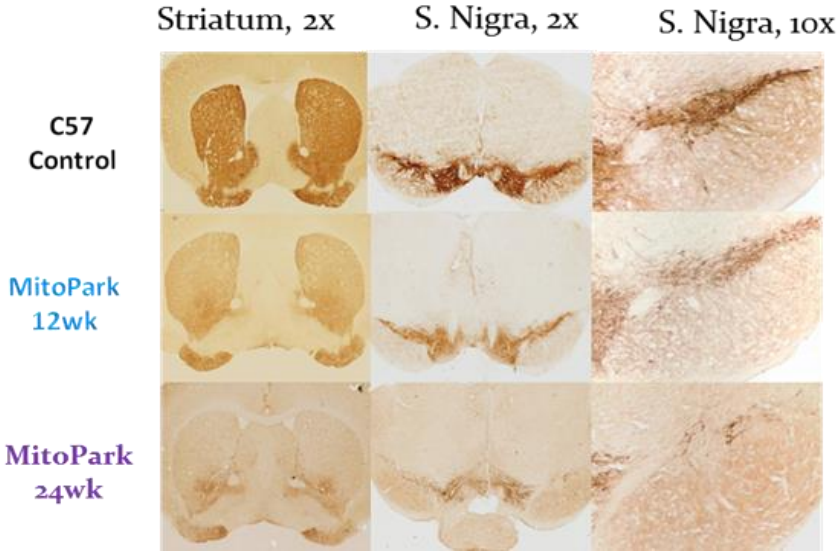


Figure 4*. Progressive neurodegeneration in MitoPark mice.*

Characterization of the nonmotor symptoms in this model was achieved in Chapter 2 of this dissertation, and identification of neuroinflammation and altered neurogenesis can be found in Chapters 4 and 5, respectively. The neuroprotective effects of mito-apocynin

(Chapter 4) and quercetin in the MitoPark mouse model were very recently published by our lab (Ay et al., 2017; Langley et al., 2017).

Background and Literature Review I:

Nonmotor Symptoms in Parkinson's disease

Prevalence and impact

Parkinson's disease can essentially be broken into 3 conceptual stages: preclinical, prodromal, and motor (Mahlknecht et al., 2015). In the preclinical phase, pathology is assumed to already be present but only identifiable by genetic, molecular or imaging biomarkers (Mahlknecht et al., 2015). The prodromal stage is defined by early nonmotor symptoms, subtle motor symptoms (Mahlknecht et al., 2015). At this stage, extranigral and nigral pathology are involved (Braak stages 1-3) and more than half of dopaminergic cells are lost (Mahlknecht et al., 2015). Final, classical motor manifestations are evident in Phase 3, which include Braak stages 3-6 (Braak et al., 2003a; Mahlknecht et al., 2015). More nonmotor symptoms present due to an extension of PD pathology (Mahlknecht et al., 2015). Nonmotor symptoms substantially contribute to reduced quality of life and increased cost of healthcare for PD patients (Chen, 2010; Kwon et al., 2016; Martinez-Martin et al., 2012).

More than 70% of PD patients present nonmotor symptoms, according to a recent cross-sectional observational study (Zhang et al., 2016b). Onset of the disease in areas of the brain outside of the substantia nigra (SN) or even peripheral onset of PD are supported by the idea that PD patients experience a variety of nonmotor symptoms before classical motor signs are observed (Mahlknecht et al., 2015). Certain symptoms are now considered to be early warning signs of PD, including hyposmia, constipation, rapid eye movement behavior

disorder, and depression (Abbott et al., 2001; Ishihara and Brayne, 2006; Kang et al., 2016; Mahlknecht et al., 2015; Postuma et al., 2015; Zhang et al., 2016b). Although nonmotor symptoms occur prior to motor deficits in typical PD, recent studies suggest this is not true for those with young onset age of PD (Zhang et al., 2016b).

There is large overlap between nonmotor symptoms. For example, patients with hyposmia and Rapid Eye Movement (REM) behavior disorder (RBD) are more likely to also exhibit cognitive deficits (Postuma et al., 2015). Both olfactory deficits and RBD were more prone to occur in patients with an older disease onset age, and in such patients, these nonmotor symptoms were likely to occur prior to development of motor symptoms (Zhang et al., 2016b).

Hyposmia

The olfactory system provides meaningful information from our environment including determining palatability of food, dangerous fumes, and other indicators of toxins (Doty, 2012). The prevalence of reduced ability to smell is about 90% in sporadic PD cases and 68% of patients surveyed described alterations in their quality of life due to impaired olfactory function (Doty, 2012). Olfactory dysfunction was more found to be more prevalent in patients with older age and in those who also had co-morbidities including depression, sleep, and autonomic dysfunctions (Zhang et al., 2016b). Olfactory disturbances do not occur in all neurodegenerative diseases, making this nonmotor symptom particularly valuable in differential diagnosis (Hawkes and Doty, 2009).

Toxins can enter the brain via the nasal epithelium and cause neuroinflammatory changes in the olfactory bulb (Doty, 2012). For instance, a recent study identified amoeboid

microglia in the anterior olfactory nucleus of AD and PD patients and increased microglia density (Doorn et al., 2014). A very recent study used axonal tracing to show the presence of a direct nigro-olfactory projection (Hoglinger et al., 2015). Moreover, injection of MPTP and 6-OHDA into the olfactory bulb resulted in decreased dopaminergic neurons in the SN (Hoglinger et al., 2015). Olfactory dysfunction was induced by dopaminergic deafferentation and was reversed by treatment with the dopamine receptor agonist, rotigotine (Hoglinger et al., 2015). These findings provide an important link between environmental toxin exposure (such as pesticide or welding fume inhalation), hyposmia, and nigrostriatal pathology in PD and related disorders (Hoglinger et al., 2015).

Many commonalities also exist between environmental risk factors for PD and smell loss; some of which include age, head trauma, and exposures to certain metals, viruses, and pesticides (Doty, 2012). Manganese exposure in airborne occupational settings has been associated with loss of smell and 3-10 fold increased risk of developing PD (Antunes et al., 2007; Doty, 2012; Tallkvist and Tjalve, 1997; Thompson et al., 2007).

Alpha-synuclein pathology in the brain first occurs in the olfactory bulb, anterior olfactory nucleus, and complexes of the glossopharyngeal and vagus nerves (Doty, 2012). Correlations between olfactory test scores and DAT levels in the striatum and hippocampus have been reported in early PD (Bohnen et al., 2008; Siderowf et al., 2005). Olfactory function is normal or only slightly impaired in PINK1, PARK2, and SNCA genetic forms of PD, but LRRK2 and GBA forms resemble idiopathic PD olfactory dysfunction (Alcalay et al., 2011; Bostantjopoulou et al., 2001; Doty, 2012; Ferraris et al., 2009; Silveira-Moriyama et al., 2010).

Mesolimbic regions such as the olfactory tubercle receive dopaminergic input from the ventral tegmental area (VTA). Dopaminergic cells can be found in the periglomerular cells of the olfactory bulb, but do no degenerate in PD (Doty, 2012; Huisman et al., 2008; Tong et al., 2000). Unlike the SN and VTA, tyrosine hydroxylase (TH) actually increases in the olfactory bulb (OB) of patients with PD and in PD animal models (Belzunegui et al., 2007; Doty, 2012; Huisman et al., 2008; Lelan et al., 2011; Yamada et al., 2004). In an intranasal MPTP model, dopaminergic and noradrenergic deficits were seen in the brain, but dopamine therapy does not help the olfactory deficits in PD patients (Prediger et al., 2010). Non-dopaminergic neurotransmitter systems are thought to contribute to or cause olfactory loss in PD (Doty, 2012).

Sleep disturbances

Sleep problems are one of the most common PD nonmotor symptoms, affecting as many as 90% of patients (Comella, 2003; Videnovic et al., 2014) (Videnovic and Golombek, 2013). Some of the sleep disturbances affecting PD patients include insomnia, hypersomnia, RBD, restless legs syndrome, and sleep apnea (Comella, 2003; Faludi et al., 2015; Videnovic and Golombek, 2013).

In RBD, patients violently act out their dreams due to loss of muscle atonia which normally occurs during REM sleep (Faludi et al., 2015; Postuma et al., 2015). Patients with RBD have a 30% risk of developing a neurodegenerative synucleinopathy after 3 years, and 66% chance after 7.5 years (Faludi et al., 2015; Postuma et al., 2015). Higher risk was also associated with hyposmia, color vision abnormalities, and subtle motor dysfunction; whereas, use of antidepressants decreased risk of disease conversion (Postuma et al., 2015). Because of the robust predictive value of these nonmotor symptoms, it is now considered practical to

include such patients with RBD in early neuroprotection trials for Parkinsonian therapeutics (Postuma et al., 2015). Prodromal RBD is thought to be associated with synuclein deposition in the lower brainstem (Braak et al., 2003b). The ventral visual stream is also thought to be involved in RBD (Ferrer et al., 2012; Marques et al., 2010). Certain antidepressants may also induce RBD symptoms.

The causes of sleep disturbances is largely unknown, but activity changes, effects of medication, alterations in melatonin, and neurodegeneration in extranigral regions are likely to contribute to these impairments which may begin before onset of motor symptoms (Videnovic et al., 2014). While dopamine depletion is involved in PD, hyperdopaminergia is associated with schizophrenia. Sleep problems are associated with both disorders, suggesting a role for dopamine in regulation of sleep (Dzirasa et al., 2006). 6-OHDA models have disrupted circadian rhythms that are partially reversed by L-DOPA administration (Videnovic and Golombek, 2013). Dopamine promotes wakefulness, which is why amphetamines and modafinil are proposed to be effective (Videnovic and Golombek, 2013). Further, mice with the D2 receptor knocked out showed decreased wakefulness (Qu et al., 2010; Videnovic and Golombek, 2013).

Dopamine is also involved in regulation of circadian rhythms by presence in retinal cells which respond to illumination and by affecting clock genes expression in the striatum and eye (Videnovic and Golombek, 2013). Dopamine is thought to mediate light signaling inputs to the superchiasmatic nucleus (SCN) (Videnovic et al., 2014; Willis, 2008). Oxidative stress and mitochondrial dysfunction have also been implicated in disruption of the circadian system in PD models. There is significant overlap between nonmotor symptoms in PD and consequences of circadian rhythm abnormalities, suggesting that circadian dysfunction may

even accelerate and exacerbate PD pathology and symptomology (Willison et al., 2013). Reduced serotonin transporter binding in the amygdala and sleep disorders are thought to contribute to fatigue as a nonmotor symptom of PD (Ferrer et al., 2012; Pavese et al., 2010; Wolters, 2001). Another hypothetical contributor to sleep disorders in PD is alterations in the penduculopontine nucleus (Arnulf, 2010; Scherfler et al., 2011).

Circadian rhythms regulate our 24 hr rhythmicity in behavior, feeding, hormone levels and various biological processes according to an endogenous biological clock located in the SCN.

Table 2. *Summary of Nonmotor Symptoms of PD*

Symptom	Prevalence	Braak Stage	Other pathology	Treatments
Hyposmia	90%	1	neuroinflammation in anterior olfactory nucleus, dopaminergic and/or noradrenergic deafferentation	-
Sleep *RBD* *EDS* *Circadian*	90%	2/3	ventral visual stream, dopamine depletion, reduced serotonin transporter binding, oxidative stress	Melatonin Clonazepam Modafinil*
Autonomic dysfunction *Gastrointestinal* *Cardiovascular* *Urinary*	27-85%	1 (constipation) 1 (bladder disorder)	changes in gut microbiota, dopamine depletion,	Macragol (constipation) Botulinum toxin (hypersalivation, urinary issues)
Neuropsychiatric *Depression* *Anxiety* *Psychosis* *Hallucinations* *Impulse control*	40%	2 (depression and anxiety) 6 (dementia and hallucination)	-dysregulation of monoamine neurotransmitters, gray and white matter reductions, increased BDNF in the nucleus accumbens (depressionand anxiety) -dopamine agonists or L-DOPA related (psychosis and hallucinations)	Pramiprexole Desipramine Nortriptyline
Cognitive dysfunction	25-50%	5 (cognitive decline)	Changes in monoaminergic innervation, cholinergic innervation, synaptic changes, metabolism shifts, oxidative damage, neuroinflammation	Rivastigmine

References: Doty et al, 2012; Doom et al, 2014; Bohnen et al, 2008; Videnovic and Golombek, 2013; Chaudhuri et al., 2006; Edwards et al., 1991; Poewe et al., 2008; Harach et al., 2017; Scheperjans et al., 2015; Seppi et al, 2011; Balestrino and Martinez-Martin, 2017; Ferrer, 2011; Ferrer et al., 2012; Tang et al., 2010; Hershey and Peevy, 2015.

Afferent pathways for light and other stimuli, referred to as 'zeitgebers,' help synchronize the clock to the environment, while efferent outputs affect nearly 10% of all expressed genes (Videnovic et al., 2014). Loss of circadian rhythms associated with autonomic and visual functions have been reported in PD patients. Furthermore, disruption of circadian rhythmicity negatively affects quality of sleep, cognition, mood, gastrointestinal function and other

functions which are knowns to be impaired in PD and other neurodegenerative disorders (Bishehsari et al., 2016; Videnovic et al., 2014; Willis, 2008).

Bright light exposure was associated with improved UPDRS scores and attenuation of daytime sleepiness in separate studies, and melatonin attenuated sleep related pathology in a MPTP primate model (Belaid et al., 2015; Paus et al., 2007). Melatonin and clonazepam are the most commonly prescribed treatments for RBD (Videnovic and Golombek, 2013). Many other treatments (i.e. treatment for fatigue with modafinil or gambling behavior with amantadine) are considered only investigational for treating nonmotor symptoms (Seppi et al., 2011).

Dysautonomia- altered gastrointestinal, cardiovascular, and urinary performances

Gastrointestinal issues

Constipation was found to be a predictor of PD, even when occurring 20 or more years prior to motor symptom onset (Savica et al., 2009). Constipation is estimated to affect 28-80% of PD patients (Chaudhuri et al., 2006; Edwards et al., 1991; Jost, 1997; Martinez-Martin and Deuschl, 2007; Poewe et al., 2008) . However, it is estimated that 80% of PD patients have prolonged transit time due to impaired colonic motility (Edwards et al., 1992; Jost and Schimrigk, 1991; Jost and Schrank, 1998). Constipation was 2.48 times more likely to occur in PD cases than in controls, even after adjusting for dietary, drug, and lifestyle habits (Savica et al., 2009). Another study showed that a bowel movement frequency of less than once per day increased incidence of PD by 2.7 (Postuma, 2015). Delays in gastric emptying and reduced bowel sounds may also indicate impaired gastrointestinal transit in PD patients (Bestetti et al., 2017; Hardoff et al., 2001; Knudsen et al., 2017; Ozawa et al., 2011).

Because constipation precedes motor symptoms, worsens with disease progression, and is accompanied by α-synuclein pathology in the gastrointestinal tract and vagus nerve, it is theorized that PD may actually start in the gut (Beach et al., 2010; Ferrer et al., 2012). The most α-synuclein aggregates in patients are found in spinal cord, vagus nerve, sympathetic ganglia, gastrointestinal tract, and endocrine organs (Beach et al., 2010; Ferrer et al., 2012). In the gut, the lower esophagus and submandibular glands show even more phosphorylated synuclein than colon and rectal tissues (Beach et al., 2010; Ferrer et al., 2012).

Moreover, the olfactory bulb and gut, areas with early dysfunction and synuclein related pathology, are also areas that are more readily exposed to environmental toxicants. A newer toxin-based model of PD, deemed the Dresden Parkinson model, uses chronic oral exposure of rotenone in wildtype mice to produce motor symptoms and pathological progression that closely resembles Braak stages (Braak et al., 2003b; Klingelhoefer and Reichmann, 2015).

Shifts in gut microbiota have recently been implicated in the development of neurodegenerative diseases such as AD and PD (Harach et al., 2017; Scheperjans et al., 2015). For example, the bacterial family Prevotellaceae was found to be 78% lower in fecal samples from patients with PD when compared to control, while five other families of bacteria were found in higher abundance. (Scheperjans et al., 2015). Certain lifestyle factors including smoking cigarettes and drinking coffee are associated with reduced risk of PD. This could potentially explain changes to the gut microbiota and inflammatory signaling and subsequent protein aggregation (Derkinderen et al., 2014; Hernan et al., 2001; Sherer et al., 2003).

Patients with gastrointestinal motility problems such as constipation have received therapeutic benefit from Macrogol (Seppi et al., 2011). PD patients with hypersalivation and drooling benefited the most from Botulinum toxin (Seppi et al., 2011).

Cardiovascular effects

Orthostatic hypotension is common and considered to be interrelated with cognitive deficits in PD patients (McDonald et al., 2016). Hypotension often causes patients to feel dizzy or lightheaded when standing up. Cardiovascular and other PD risk factors independently contribute to cognitive effects observed in PD patients (Jones et al., 2017).

Urinary issues

Urinary tract issues are also common in the PD population. 27-85% of PD patients have lower urinary tract symptoms such as increased frequency or urgency of urination, painful urination, and nocturia (McDonald et al., 2017). Detrusor hyperreflexia and loss of effects from basal ganglia suppression of micturition (release of urine from the bladder) contribute to the pathological phenotype (McDonald et al., 2017). Potential therapeutic strategies include anti-muscarinic drugs and botulinum toxin (McDonald et al., 2017).

Neuropsychiatric symptoms

Neuropsychiatric symptoms, primarily including anxiety and depression, affect almost half of PD patients. Similar to other nonmotor symptoms, they can occur prior to motor symptoms and worsen with increasing motor impairment. Anxiety and depression are the nonmotor symptoms which have the most significant effect on patients quality of life, as determined by health related quality of life scores (HRQoL) (Balestrino and Martinez-

Martin, 2017). Many neuropsychiatric symptoms such as depression and anxiety are associated with sleep disturbances, further impairing quality of life in PD patients (Crane et al., 1983).

Depression

Prevalence of major depressive disorder in PD is 17%, while minor depression is 22% (Balestrino and Martinez-Martin, 2017; Reijnders et al., 2009). These ranges reflect the heterogeneity in diagnostic criteria and methods of assessment for depression. Dysthymia, a chronic mood disorder similar to depression but less severe, has 13% prevalence in PD (Balestrino and Martinez-Martin, 2017; Reijnders et al., 2009), while 39.8% of patients have apathy (Balestrino and Martinez-Martin, 2017; den Brok et al., 2015). Because depression can precede motor symptoms, it has been considered as either a risk factor or an early symptomatic predictor of PD (Blonder and Slevin, 2011; Leentjens et al., 2003; Nilsson et al., 2001; Nilsson et al., 2002; Schuurman et al., 2002). DSM criteria may lead to under-diagnosis of depression and dysthymia due to exclusion criteria (Blonder and Slevin, 2011).

Dopamine is central to the brain's reward system pathways, and may certainly play a role in the depression and cognitive changes observed in PD (Blonder and Slevin, 2011; Elliott et al., 2003; Le Foll et al., 2005). Depressive symptoms in PD are thought to be the result of catecholamine and serotonin depletion or dysregulation, particularly in mesolimbic and mesocortical pathways (Blonder and Slevin, 2011; Elliott et al., 2003; Le Foll et al., 2005). Serotonin is now understood to modulate nigrostriatal, mesolimbic, and neocortical dopaminergic pathways (Alex and Pehek, 2007; Blonder and Slevin, 2011; Di Matteo et al., 2008). Frisina et al. (2008) showed that catecholaminergic regions have a higher prevalence

of pathological features, including neuronal loss and gliosis, in depressed PD patients than non-depressed PD patient brains (Frisina et al., 2009).

Dysregulation of monoamine neurotransmitters, hypoperfusion, and gray and white matter reductions have been reported. Such changes are typically seen in the brainstem (raphae and locus coeruleus), caudate, thalamus, limbic system, anterior cingulate, and medial frontal cortex in patients with Parkinsonian depression (Blonder and Slevin, 2011). PET, MRI, and TCS can be useful in identifying abnormalities in the basal ganglia and limbic system (Blonder and Slevin, 2011). Increased BDNF-TrkB signaling and synaptogenesis in the NAc by deletion of α7 nAChR plays a key role in depression (De Vry et al., 2016; Han et al., 2016; Ma et al., 2016; Silva et al., 2016; Zhang et al., 2016a).

Drugs considered efficacious for depressive symptoms of PD include dopamine agonist pramipexole, tricyclic antidepressants nortriptyline and desipramine (Seppi et al., 2011). Although SSRIs such as fluoxetine are now the most commonly used drugs for treating depression in the general population, recent recommendations suggest there is insufficient evidence to warrant their use in PD patients (Seppi et al., 2011). The effect of L-DOPA therapy or dopamine agonists on depressive-like behavior in the MitoPark mice and other models should also be examined, due to clinical studies suggesting that stimulation of dopamine D2-like receptors has antidepressant response (Blonder and Slevin, 2011; Gershon et al., 2007; Rana and Galani, 2014).

Anxiety

Studies suggest that approximately 40% of all PD patients have anxiety symptoms including social phobia, generalized anxiety, and panic disorder (Dissanayaka et al., 2010;

Negre-Pages et al., 2010; Pontone et al., 2009; Stein et al., 1990). Anxiety symptoms are more common during the "off" state, and fluctuate similarly to motor symptoms (Blonder and Slevin, 2011; Witjas et al., 2007; Witjas et al., 2002). The Beck Anxiety Inventory (BAI) or Geriatric Anxiety Inventory (GAI) are typically used to screen patients for anxiety, but there is unfortunately overlap of features with PD motor symptoms, such as trembling hands and rigidity (Salazar et al., 2017).

Many times, anxiety and depression symptoms go hand in hand. Naturally, the associated pathology also mirrors depression causes- monoamine neurotransmitter abnormalities (Blonder and Slevin, 2011; Remy et al., 2005; Roy-Byrne et al., 1986; Stein et al., 1990). A few recent cross-sectional analyses showed lower uptake of DAT in the caudate nucleus is associated with a higher level of anxiety, strengthening the correlation between dopaminergic dysfunction and anxious phenotype (Erro et al., 2012; Picillo et al., 2017).

Other neuropsychiatric symptoms in PD

Although not as common, neuropsychiatric symptoms of PD other than anxiety and depression occur in PD patients. For example, psychotic symptoms were estimated to occur in 16-75% of patients, while minor psychotic symptoms were found in 17-72% (Balestrino and Martinez-Martin, 2017; Chang and Fox, 2016; Fenelon and Alves, 2010). Impulse control disorders are estimated to affect 6-18% of PD patients (Balestrino and Martinez-Martin, 2017; Zhang et al., 2014). Visual hallucinations were more commonly reported than auditory, with prevalence's of 22-38% and 0-22%, respectively (Balestrino and Martinez-Martin, 2017; Chang and Fox, 2016; Fenelon and Alves, 2010). Lewy bodies in the amygdala and cortex correlated with hallucinations in a few different PD studies (Ferrer et

al., 2012; Harding et al., 2002; Papapetropoulos, 2006; Yamamoto et al., 2007). Delusions are estimated to affect 5% of PD patients (Balestrino and Martinez-Martin, 2017; Chang and Fox, 2016; Fenelon and Alves, 2010).

Compulsive behaviors may affect up to 14 % of PD patients receiving D3 receptor agonists (Dodd et al., 2005; Suzuki et al., 1998; Voon et al., 2007). Some of these included gambling, hypersexuality, compulsive shopping, and overeating (Blonder and Slevin, 2011; Dodd et al., 2005; Voon et al., 2007). Clozapine is clinically useful for treating PD-related psychosis (Seppi et al., 2011).

Cognitive dysfunction

Execute functions like working memory and visuospatial capacity are most commonly affected in the cognitive deficits of PD. Co-morbidity with neuropsychiatric disorders like anxiety and depression or sleep problems are quite common (Kulisevsky et al., 2008). Although commonly used, spatial learning tests such as Morris water maze, Barnes maze, and novel object recognition may not actually be the most relevant tests for PD-like cognitive deficits. Short-term memory and cognitive flexibility tests would be more relevant to the dysfunction typically described in PD patients. The effects of anxiety-like behavior and motor deficits could also impact performance in certain cognitive tests (Halliday et al., 2014). Spatial learning and memory is thought to involve neurogenesis in the dorsal hippocampus, while regulation of emotion is believed to occur in the ventral hippocampus (Sahay et al., 2007; Sahay and Hen, 2007). Fear-conditioning based cognitive processes are independent of hippocampal activity (Pugh et al., 1998).

A number of factors leading to cortical changes and consequent cognitive deficits are proposed. Some of these include monoaminergic innervation, cholinergic innervation, synaptic changes, metabolism shifts, consequences of oxidative damage, and transcriptional or protein expression changes (Ferrer, 2011; Ferrer et al., 2012; Tang et al., 2010). Both dopaminergic and cholinergic deficiencies, or a combination of the two, have been implicated in cognitive dysfunction in PD (Calabresi et al., 2006; Ferrer et al., 2012). These neurochemical changes could potentially be mediated by α-synuclein aggregation impairing synaptic function (Dalfo et al., 2004; Ferrer et al., 2012; Nemani et al., 2010).

Impaired cortical metabolism was shown to play a role in the pathophysiology of cognitive deficits in PD, as glucose metabolism was reduced (Ferrer et al., 2012; Yong et al., 2007). Multiple neurotransmitter impairments contribute to the cognitive deficits associated with PD, as demonstrated by various animal models (Halliday et al., 2014). Parkin knockout mice were evaluated for motor function, olfactory discrimination, anxiety, and depressive-like behavior, but did not show significant differences versus control mice (Rial et al., 2014). Procedural and short-term spatial memory tasks were impaired and deficiencies in hippocampal plasticity were also observed (Rial et al., 2014).

Unlike other symptoms, studies have shown that there is no significant correlation between Braak stage and severity of cognitive impairments in advanced PD (Ferrer et al., 2012; Jellinger, 2009a, b; Parkkinen et al., 2008; Parkkinen et al., 2005; Weisman et al., 2007). Parkinson's disease dementia (PDD) is thought to arise from dopamine depletion combined with α-synuclein and Tau pathologies, and loss of limbic and cortical neurons. PD with mild cognitive impairment is mirrored by low dose MPTP treatment in primates

(Halliday et al., 2014). Vascular changes are common in PD patients and may be associated with the observed reduction in cognitive function (Ferrer et al., 2012; Jellinger, 2010).

Inflammation disrupts cognitive function through altering levels of neurotrophic factors in the brain such as brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), and insulin-like growth factor (IGF), which are known to be critically involved in supporting memory formation, neurogenesis and long term potentiation (Fan et al., 1995; Liu et al., 2006; Shoemaker et al., 1990). Oxidative stress in the hippocampus can lead to memory deficits by impairing synaptic plasticity (Lee et al., 2015). Stress also decreases proliferating cells in the dentate gyrus (Schoenfeld and Gould, 2013).

Environmental enrichment and exercise produce more new neurons in the hippocampus and improve learning and memory (Uda et al., 2006). Exercise has also been shown to increase overall brain plasticity and alleviate depression (Cotman et al., 2007). Many antidepressants enhance neurogenesis (Cotman et al., 2007). The cholinergic system has been implicated in cognitive impairment, and normal cholinergic activity contributes to hippocampal neurogenesis via the CREB/BDNF pathway. To this end, acetylcholinersterase (AChE) inhibitors are the primary treatment for patients with memory deficits (Bohnen and Albin, 2011; Lee et al., 2015). Rivastigmine is the only acetylcholinesterase inhibitor with sufficient evidence to be considered efficacious in treating dementia in PD patients (Seppi et al., 2011).

Adult neurogenesis

Previously, it was a long held belief that the mammalian brain did not grow new neurons, but in the past decade much research has been done to clarify the locations and functions of adult born neurons in the brain (Aimone et al., 2010; Batiz et al., 2015; Deng et al., 2010; Lim and Alvarez-Buylla, 2016). It is now understood that new NSCs are made every day in the subventricular zone (SVZ), which lines the lateral ventricles, and the subgranular zone (SGZ) of the dentate gyrus (DG) in the adult mammalian brain (Biebl et al., 2000). Adult NSCs have the capacity to differentiate into neurons, allowing for adaptation to stimuli in our environment (Holmes, 2016; Kempermann, 2015; LaDage, 2015). In the adult mouse SVZ, type B astrocytic stem cells give rise to the rapidly dividing type C transit-amplifying progenitor (TAP) cells, which then produce type A neuroblasts (Wang et al., 2011). These neuroblasts migrate along the rostral migratory steam (RMS) tangentially in a chain to the olfactory bulb (OB) where they migrate radially to form olfactory interneurons in the glomerular or periglomerular layer (Dohi et al., 2010). This specialized neuroblast migration was also shown to occur in humans in the ventriculo-olfactory neurogenic system (Curtis et al., 2007). Although, because of the enlarged frontal cortex in humans, the path shifts caudally prior to reaching the olfactory tract (Curtis et al., 2007). While ratio of A:B:C type cells is 3:2:1 in rodents, humans have a 1:3:1 ratio which is attributed to the lesser importance of olfaction in

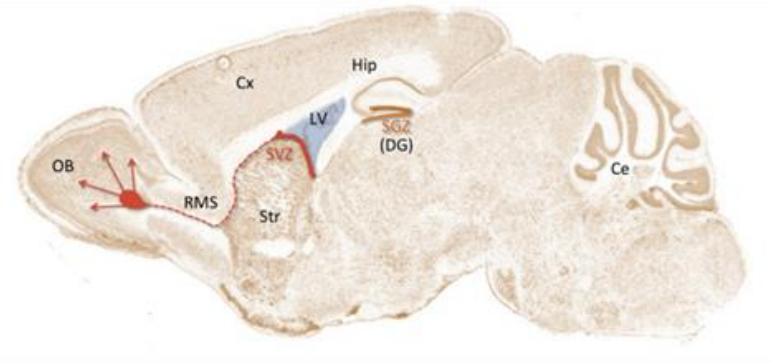


Figure 5. *Neurogenic regions of the brain. Adapted from Batiz et al., 2015.*

humans versus mice (Doetsch et al., 1997). The function of this pathway in olfactory discrimination is most easily understood by loss of function studies, wherein a loss of olfactory bulb neurogenesis correlates with impaired olfaction in rodents (Doetsch et al., 1997; Mobley et al., 2014).

Adult progenitor cells also reside in the hilar side of the granule cell layer in the SGZ of the dentate gyrus (Aimone et al., 2010). Here, type 1 cells, described as slow dividing or quiescent, generate TAPs, which in turn differentiate into either granule cells with glutamatergic inputs or astrocytes (Kempermann et al., 2004; Peretto and Paredes, 2014). Radial and horizontal type 1 NSCs differ in that horizontal cells appear to divide more quickly. There are a few different types of transit amplifying progenitor cells in the SGZ which mostly differ in morphology and marker expression. For example, type 2a TAPs are DCX-negative but type 2b are DCX-positive, while both express Nestin. Nestin negative type 3 cells, also referred to as neuroblasts, are also DCX+. Unlike in the SVZ/RMS pathway which is responsible for olfactory discrimination, adult hippocampal neurogenesis is associated with learning, memory, and neuropsychiatric symptoms such as depression and anxiety (Apple et al., 2016; Lee and Son, 2009; Ortega-Martinez, 2015; Pereira Dias et al., 2014). Interestingly, many antidepressants (i.e. amitriptyline, desipramine, lithium) or activities known to prevent depression (i.e. exercise, flavonoid intake, environmental enrichment) increase hippocampal neurogenesis, while stress downregulates neurogenesis (Apple et al., 2016; Haase and Brown, 2015; Hussaini et al., 2014; Jessberger and Gage, 2008; Miller and Hen, 2015; Ryan and Nolan, 2016; Warner-Schmidt and Duman, 2006).

Regulation of neurogenesis is a complex process and may involve a variety of transcription factors, neurotrophic factors, neurotransmitters, hormones, cell signaling

pathways, and epigenetic mechanisms (Hsieh, 2012; Hsieh and Eisch, 2010; Li and Zhao, 2008; Mahmoud et al., 2016; Palm et al., 2013). Serotonergic innervation from the raphe nucleus increases neurogenesis and proliferation in both the SVZ and SGZ, whereas dopaminergic innervation from the SN and ventral tegmental area increase proliferation in the SVZ through D2-like receptors (Chaudhury et al., 2015; Gordon and Goelman, 2016; Haase and Brown, 2015). Additionally, GABA increases differentiation, decreases neurogenesis, and can increase or decrease progenitor cell migration depending on receptor subtype in the SGZ (Abe et al., 2016; Haase and Brown, 2015). Some key pathways that have been shown to regulate adult neurogenesis include cAMP-CREB, p53, TGFβ, Wnt/β-catenin, PI3K/Akt and ERK signaling (Daynac et al., 2016; Fujimaki et al., 2015; Medrano and Scrable, 2005; Ortega-Martinez, 2015; Palm et al., 2013; Shioda et al., 2009). Recent studies suggest that antidepressant-induced neurogenic activity may be dependent on the glucocorticoid receptor and PKA signaling (Apple et al., 2016). Neuroinflammation strongly inhibits neurogenesis and contributes to cognitive deficits associated with neurodegenerative diseases (Ekdahl et al., 2003; Monje et al., 2003; Ryan and Nolan, 2016).

Whereas almost ten thousand new neurons are generated daily in mice, only about 700 per day are estimated to be produced in humans, based on C14 dating from the Cold War (Ernst and Frisen, 2015). Olfactory bulb neurogenesis is considered to be very limited or even completely absent in humans, making it an unlikely cause of hyposmia in PD (Ernst and Frisen, 2015). Although there is a substantial amount of hippocampal neurogenesis in adult humans, subventricular zone derived neural stem cells generate new interneurons to the striatum instead of migrating to the olfactory bulb (Bergmann et al., 2015). Striatal neurogenesis in humans is considered to play a role in cognition and motor function, but not

olfaction (Bergmann et al., 2015). In mice, it takes several days to weeks for a newborn cell to mature and functionally integrate (Kempermann and Neumann, 2003; van Praag et al., 2002), whereas in macaque monkeys it takes approximately 6 months (Kohler et al., 2016).

Recent findings argue the true source of neural progenitor cells may even be a part of a newly discovered CNS lymphatic system (Harzsch and von Bohlen Und Halbach, 2016) (Louveau et al., 2015). Inflammation results in production of cytokines, nitrative and oxidative stress which inhibit mitochondrial function in neural progenitor cells. Severe mitochondrial damage may then lead to cell death. Differentiation of progenitor cells requires a lot of ATP due to growth of neuronal processes, cytoskeletal remodeling and organelle transport, so mitochondrial function is essential (Kohman and Rhodes, 2013). Microglial activation directly correlates with impaired neurogenesis in irradiated animals, while anti-inflammatory drugs reverse impairment (Ekdahl et al., 2003; Mizumatsu et al., 2003; Monje et al., 2003). Alternatively activated microglia are thought to support neurogenesis.

Mitochondrial function is essential to neural stem cell survival and differentiation. While various cell lines, like HeLa, XP30RO and GM10115, can tolerate mitochondrial DNA (mtDNA) depletion for extended time periods, neural precursor cells die within a short time after mtDNA depletion (Fike et al., 2009). A few mitochondrial transcription factors, NeuroD6 and Bcl-xl are also important for neuronal precursor differentiation (Doeppner et al., 2009; Grisolia et al., 2009). Apoptosis in SVZ neuroblasts was found to be independent of DAT and other transporters in a MPTP model, since inhibitors showed no effect at reducing toxicity. This suggests that some other novel pathway mediates MPTPs effects on neurogenesis (Shibui et al., 2009).

Dopamine promotes proliferation of neural progenitor cells in the SVZ through releasing EGF in a PKC-dependent manner and consequently activating EGFR (O'Keeffe et al., 2009b). Furthermore, fewer EGFR+ cells were found in the SVZ of human PD patients when compared to age-matched controls (O'Keeffe et al., 2009b). EGR1 regulates EGFR-mediated NSC and NPC proliferation (Alagappan et al., 2013). Hypoxic conditions up-regulate EGR1 through the ERK pathway, and further activate EGFR (Maegawa et al., 2009; Nishi et al., 2002).

Neurogenesis in neurological disorders

Although the adult brain is particularly vulnerable to toxic or mechanical insults, neural progenitor cells provide a possibility of repairing damaged tissue by being recruited to the site of injury. Furthermore, increasing evidence indicates that following acute neuropathology such as stroke, traumatic brain injury, seizure, or certain neurodegenerative conditions, there is a proliferation of adult NSCs (Mouhieddine et al., 2014; Nakatomi et al., 2002; Parent, 2007; Rolfe and Sun, 2015; Xiong et al., 2010). Psychiatric and cognitive disorders have been behaviorally and biochemically linked to neurogenesis, mainly due to the regulatory effects of monoamines and neurogenic effects of antidepressants (Apple et al., 2016; Haase and Brown, 2015). Subsequent to ischemia or brain trauma, increased neurogenesis and proliferation of adult NSCs in areas not associated with neurogenesis can be found, yet it is unclear why spontaneous stroke-induced or injury-induced neurogenesis does not lead to functional recovery (Lambert et al., 2016; Lindvall and Kokaia, 2015; Marlier et al., 2015; Rolfe and Sun, 2015; Sun, 2014). Because this NSC regeneration is at an insufficient capacity to reverse these disease processes, enhancing the environment with growth factors to further promote proliferation and differentiation provides a more promising

approach to neuronal replacement (Foltynie, 2015; Guo et al., 2016a; Paillard et al., 2015; Zhang et al., 2013).

Interestingly, many proteins involved in the pathogenesis of neurodegenerative diseases play roles in neurogenesis and synaptic plasticity (Jamwal and Kumar, 2015; Lazarov and Marr, 2010; Winner et al., 2011; Winner et al., 2012). Also, aging is associated with a reduction in adult neurogenesis and accompanying cognitive deterioration in the elderly, and is a key risk factor for neurodegenerative diseases (Rodriguez-Nogales et al., 2016; Seib and Martin-Villalba, 2015). In mouse models of Huntington's disease, there is primarily a decrease in proliferation the DG and decreased neuronal differentiation in the OB (Gil-Mohapel et al., 2011; Kohl et al., 2010). Although no clear change in SVZ proliferation was observed in mouse models, human studies suggest an increase in SVZ neurogenesis may occur in Huntington's Disease (Curtis et al., 2005; Kohl et al., 2010). Similarly, changes in neurogenesis in both neurogenic niches have been observed in transgenic Alzheimer's disease (AD) models and human AD, although it is still unclear whether a decrease or compensatory increase in neurogenesis is occurring due to variation in models, markers, and study design (Lazarov and Marr, 2010; Marlatt and Lucassen, 2010; Winner et al., 2011).

In transgenic mouse models of PD, overexpression of human wild type α-synuclein or A53T mutation resulted in a reduction of neurogenesis in the DG, SVZ, and OB (Winner et al., 2004; Winner et al., 2008). Most studies using lesion-based PD models, such as 6-OHDA or MPTP toxin treated rodents, also reported decreased neurogenesis in the SVZ, although in some studies results were contradictory or inconclusive (He and Nakayama, 2015; O'Keeffe et al., 2009a; Peng and Andersen, 2011). It has been recently reported that L-DOPA and pramipexole, a dopamine agonist, were able to attenuate alterations in adult

neurogenesis in the DG and OB, and restore corresponding nonmotor behavioral changes in bilateral 6-OHDA lesioned mouse (Chiu et al., 2015). In human PD, a number of studies indicate deregulated adult neurogenesis as evidenced by fewer PCNA+ NSCS in the SVZ, Nestin+ NPCs in the OB, and EGFR+ cells in the SVZ of PD brains when compared to controls which may be partially restored by chronic L-DOPA treatment (Hoglinger et al., 2004; O'Keeffe et al., 2009b; O'Sullivan et al., 2011). However, other groups have argued that the proliferative capacity of the SVZ is maintained in the PD brain (van den Berge et al., 2011). Importantly, PD associated genes have been shown to play roles in embryonic and adult neurogenesis, neuronal development, and expression of neurotrophic factors, substantiating the idea PD may even be regarded as a developmental disorder (Berwick and Harvey, 2013; Garcia-Reitboeck et al., 2013; George et al., 2007; Jiang et al., 2012; Le Grand et al., 2015; Lee et al., 2013; Li et al., 2012b; O'Keeffe et al., 2008; Zhang et al., 2012).

Background and Literature Review II:

Novel Therapeutic Strategies in PD

Current treatments

There is no cure for Parkinson's disease (PD), and current treatments simply address the symptoms instead of stopping the neurons from degenerating. L-3-4-dihydroxyphenylalanin (L-DOPA) is considered the gold standard for PD treatment, and has been used successfully for half a century now (Espay et al., 2017). L-DOPA can lead to many side effects including dyskinesia at peak dose, nausea, hypotension, and anxiety (Espay et al., 2017). To avoid adverse side effects caused by peripheral dopamine synthesis from L-DOPA, it is typically administered alongside a DOPA decarboxylase inhibitor (DDCI) such as carbidopa. Extended release L-DOPA/carbidopa formulations and intestinal infusions are being explored to reach target plasma levels of the drugs and avoid undesirable effects during the "off" period (Buhmann et al., 2017; Espay et al., 2017).

Adjunct therapy with monoamine oxidase (MAO-B) and catechol-O-methyltransferase (COMT) inhibitors is also used to avoid dopamine degradation (Muller, 2012; Smith et al., 2012). Dopamine agonists are often considered as a first line of defense in early PD patients, since the effectiveness of L-DOPA wears off after the 3-5 year "honeymoon period" (Hisahara and Shimohama, 2011; Oertel and Schulz, 2016). However, it is not recommended in older PD patients, due to cognitive and neuropsychiatric effects (Oertel and Schulz, 2016; Poewe et al., 2008). Deep brain stimulation in the subthalamic nucleus, ventral intermediate nucleus, and globus pallidus following surgically placed electrodes is also beneficial to motor symptoms in PD patients (Deuschl et al., 2013; McIntyre and Anderson, 2016). Disease

modifying therapies currently in clinical trials include antioxidants, anti-inflammatory, neurotrophic factors and some repurposed compounds. Drugs are also under study in clinical trials for targeting nonmotor symptoms including constipation, swallowing difficulties, impulse control, and cognitive decline.

Mitochondria-targeted antioxidants

Although genetic and environmental factors may increase individual risk to idiopathic PD, the exact etiology remains unknown. It is thought that a number of genetic mutations and environmental factors converge on central pathophysiological pathways, ultimately leading to dopaminergic neuronal loss (Bellou et al., 2016; Mandemakers et al., 2007; Ritz et al., 2016). Oxidative damage to proteins, nucleic acids, and lipids is evident in post-mortem ventral mesencephalic tissue from PD patients and animal models of the disease (Bosco et al., 2006; Ghosh et al., 2016; Langley, 2017). Furthermore, key antioxidant mechanisms may be impaired (Jin et al., 2014; Sian et al., 1994). Dopamine oxidation, either enzymatic (monoamine oxidase A) or non-enzymatic, can also lead to hydrogen peroxide formation, further contributing to oxidative stress in the SN (Graham et al., 1978; Hermida-Ameijeiras et al., 2004). High iron content in the SN allows for conversion of hydrogen peroxide to the hydroxyl radical via Fenton's reaction (Nappi and Vass, 1997). The highly toxic hydroxyl radical may form DNA damage in the form of the oxidized metabolite 8-hydroxyguanosine (8-OHG), which has been observed in cerebrospinal fluid of PD patients (Cerchiaro et al., 2009; Isobe et al., 2010).

As the ATP producing powerhouse of the cell, the mitochondrion supplies energy through oxidative phosphorylation, particularly in the brain where there is high energetic

demands. This process in not 100% efficient though, with multiple sites of the electron transport

chain leaking reactive oxygen species (ROS), mainly in the form of superoxide anion from Complex I or III (Jin et al., 2014; Turrens, 2003). Many suitable models for studying PD make use of mitochondrial complex inhibition to increase ROS and resulting cell death (Charli et al., 2016; Ghosh et al., 2016). Given that mitochondrial dysfunction and oxidative stress are considered to be major contributing factors in PD pathology, recent studies have focused on developing mitochondrially-targeted antioxidants to ameliorate these effects (Ghosh et al., 2016; Jin et al., 2014; Langley, 2017).

Traditional antioxidants have largely been unsuccessful in clinical studies due to their failure to reach the mitochondria in sufficient quantity, limited brain bioavailability, or adverse side effects. Various anti-oxidants have been tested in different animal models of PD with little success (Golden and Patel, 2009; Jin et al., 2014; Kaur et al., 2003). Modification of small molecules can be performed to target the drug to specific organelles, and such strategies have resulting in promising new potential PD therapeutic drugs (Ghosh et al., 2016; Jin et al., 2014). Vitamins have been explored as a therapeutic approach due to their oxidative stress scavenging effects and neuroprotective properties (Hughes et al., 2016; Sutachan et al., 2012). Epidemiology studies suggest that a combination of vitamin e and beta carotene lowers the PD risk (Miyake et al., 2011), but more data is needed before making any conclusive remarks about dietary vitamin supplementation and PD risk due to the large data gaps and inconsistent results between studies (Hughes et al., 2016; Zhu et al., 2017). A popular source of dietary antioxidants is the resveratrol in red wine, teas, and vegetables. Resveratrols effect is thought to be due to sirtuin 1 (SIRT1) activation (Guo et al.,

2016b; Sun et al., 2010; Zeng et al., 2017). Another natural antioxidant, quercetin, is abundantly found in fruits and vegetables and was recently shown by our laboratory to be neuroprotective in the severe, progressive MitoPark model of PD (Ay et al., 2017; El-Horany et al., 2016; Lee et al., 2016b; Shah and Duda, 2015). Apocynin, another plant based antioxidant structurally related to vanilla, and its dimer were found to be effective at reducing neuroinflammation and neurodegeneration in the MPTP toxin-based model and a transgenic LRRK2 model, but the high doses used in the studies (300 mg/kg) warranted further research to identify more efficacious molecules (Dranka et al., 2013; Ghosh et al., 2016)

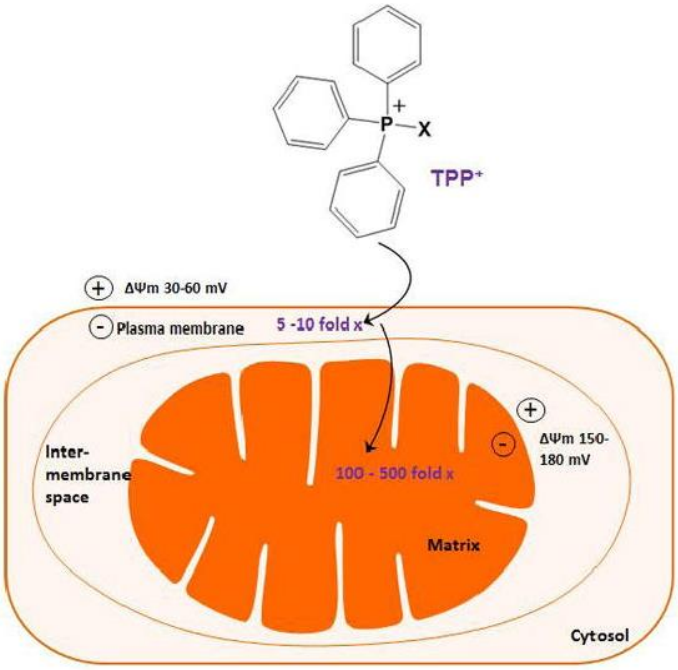


Figure 6. *TPP+ entry into the cell and mitochondrion. Adapted from Jin et al., 2014.*

Scientists have discovered two ways of modifying a small molecule to increase its affinity for the mitochondrion. First, conjugation to a lipophilic cation moiety allows it to easily pass through lipid bilayers and aim for the negative inner mitochondria (Skulachev, 2005). The other method is to incorporate it into a mitochondria-targeted peptide. Triphenylphosphonium cation was used to create a series of mitochondria target compounds, including Mito-Q_{10}, Mito-TEMPOL, and Mito-VitE (Jin et al., 2014).

Neuroinflammation

Neuroinflammation is now recognized as a significant contributor to PD pathology. Microglial and astroglial activation, secretion of proinflammaotry cytokines and nitric oxide production have all been shown in human PD cases and animal models of the disease (Kempermann and Neumann, 2003; Mogi et al., 1994; Panicker et al., 2015). About 10% of the brains' glia are microglia which typically display a ramified morphology in their resting state. Microglia remove debris and toxins, help to establish synapses during development, and produce trophic factors (Carson et al., 2007; Tansey et al., 2008). Upon stimulation by antigens, misfolded proteins, LPS, or interleukins, microglia can become activated. In the activated state, microglia morphology changes to a larger cell body with shorter, thickened processes (Panicker et al., 2015; Xiong et al., 2016). These classically activated microglia are referred to as having an amoeboid shape and are known to secrete proinflammatory factors. Another activation phenotype deemed 'alternative' activation is associated with wound healing and repair, where the cells instead produce protective factors such as IL-10 and arginase 1. Other intermediate stages of microglial activation have also been proposed (Qin et al., 2007). Microglia can also become classically activated by neuronal damage in a self-propelling process referred to as reactive microgliosis.

Astrocytes are the most abundant glia in the brain. Astrocytes migrate to the site of injury and develop a hypertrophic morphology during neurodegenerative processes (Ghosh et al., 2007; Ghosh et al., 2009). Chronic, progressive neuroinflammation is seen in PD and other neurodegenerative disorders, making a promising drug development target.

NADPH oxidase (NOX) inhibitors

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (or NOXs) are a membrane-bound, superoxide generating enzymes present on various cell types throughout the body (Cooney et al., 2013). NOXs use NADPH as an electron donor to transport electrons from flavin adenine dinucleotide (FAD) and hemes within the membrane to reduce oxygen to superoxide ($O_2^{\cdot-}$). Superoxide dismutase turns most superoxide into hydrogen peroxide (H_2O_2), which can interact with nitric oxide to form peroxynitrite (ONOO-) (Ara et al., 1998; Cotman et al., 2007; Przedborski and Vila, 2001). NOX1 and 2 are expressed in neurons, microglia, and smooth muscle cells, while NOX3 is found in the vestibular system of the inner ear. NOX4 is constitutively active and present in kidney, smooth muscle, neurons, and astrocytes (Serrander et al., 2007; Vendrov et al., 2015). More specifically, NOX4 has been shown to localize in the mitochondria of neurons (Case et al., 2013). NOX5 is primarily expressed in fetal tissues and spleen. DUOX 1 and 2 produce hydrogen peroxide and expressed in the thyroid (Cooney et al., 2013). Homologues of NOXs in the CNS play roles in memory, neuronal signaling, and development (Cristovao et al., 2012).

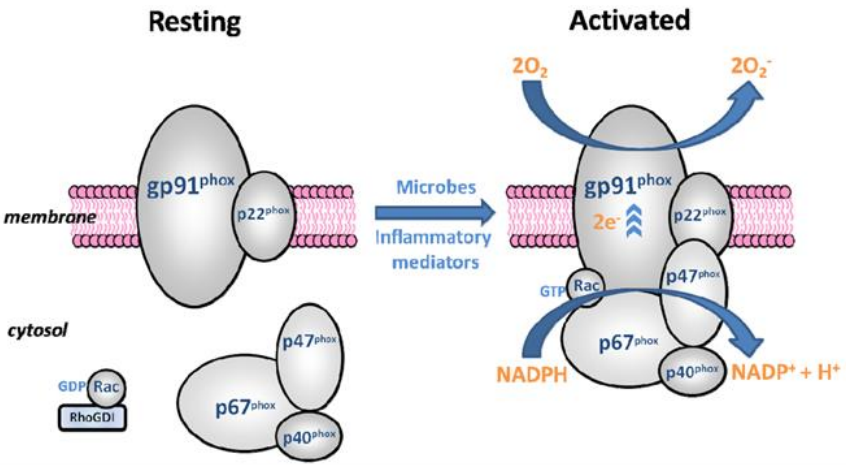


Figure 7. *NADPH oxidase subunit recruitment and activation*

Activation of NOX2 requires phosphorylation of cytosolic subunits (p40phox, p47phox, and p67phox) and their recruitment to the plasma membrane to join membrane subunits (gp91phox and p22phox) and G proteins (Babior, 2000). In activated microglia cells, this superoxide production can lead to damage to neighboring dopaminergic neurons (Block and Hong, 2005; Cristovao et al., 2012; Gao et al., 2003; Wu et al., 2005) In neurons, NOX1, NOX2, and NOX4 isotypes were found to be expressed following traumatic brain injury, but NOX2 was the most sensitive in responding to injury (Cooney et al., 2013). NOX2 was also up-regulated in models of multiple sclerosis (MS), ischemia, spinal cord injury, and PD (Cooney et al., 2013; Ghosh et al., 2016). Recent research suggests that NOX activation and mitochondrial ROS may activate each other in a feed-forward cycle (Cristovao et al., 2012; Dikalov, 2011). Genetic deletion of various NOX isoforms, particularly NOX1 and NOX2, has neuroprotective effects in various animal models of disease including PD (Seredenina et al., 2016; Wu et al., 2003; Zhang et al., 2004).

Unlike genetic knockout models, most pharmacological interventions targeting NOXs have limited isoform specificity and exert free radical scavenging properties as well (Altenhofer et al., 2015). A peptide, Gp91ds-tat, inhibits NOX2 by binding directly to impair its activity, but has limited oral bioavailability (Altenhofer et al., 2015). A small molecule, GSK2795039, is a reversible, direct inhibitor of NOX2 that reaches the brain (Hirano et al., 2015). A recently designed NOX1/4 inhibitor, GKT137831 has recently made it to phase II clinical trials (Altenhofer et al., 2015; Hirano et al., 2015).

Mito-apocynin

Apocynin is from the plant *Picrorhiza kurroa* and is well studied in various models of disease for its NOX2 inhibiting properties, ability to cross the blood-brain-barrier, and tolerability (Lull et al., 2011; Trumbull et al., 2012) . Apocynin is from the plant *Picrorhiza kurroa* and is well studied in various models of disease for its NOX2 inhibiting properties, ability to cross the blood-brain-barrier, and tolerability (Lull et al., 2011; Trumbull et al., 2012).

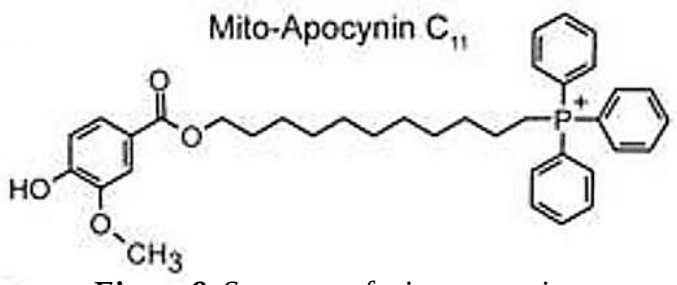


Figure 8. *Structure of mito-apocynin.*

NOX2 inhibitors apocynin (4-hydroxy-3-methoxyacetophenone) and diapocynin were recently found to be neuroprotective in MPTP and LRRK2R1441G mouse models of PD (Dranka et al., 2013; Ghosh et al., 2012)**.** Because high doses were used in the apocynin and diapocynin studies (300 mg/kg), more efficacious analogs were designed to be mitochondria targeted. Another MPTP mouse study from our group showed that Mito-apo-C_2 protects against neuroinflammation and neurodegeneration in the MPTP model (Ghosh et al., 2016)**.** In Chapter 4, we evaluate the disease-modifying efficacy of Mito-apo-C_{11} in the MitoPark model.

Alternative Therapeutic strategies

Dopaminergic differentiation of transplanted NSCs

Although no stem cell therapy is currently approved for clinical use yet, a number of therapies are being tested for application in various neurodegenerative disorders including

macular degeneration, MS, and PD (Levy et al., 2016). Of all the neurological disorders, PD seems to be the most promising candidate for stem cell transplantation success so far, with extensive research in animal models and clinical trials (Levy et al., 2016). Patient derived neural stem cells can also be useful in studying individual responsiveness to various therapeutic targets *in vitro* to better understand the mechanism of the disease in that specific patient (Cooper et al., 2012). The first transplantation of fetal nigral cells into the rat brain was in 1972 (Echavarria-Mage et al., 1972). Since then, advances have been made in both the sources of tissue and scaffoldings, leading to recent studies which used a 3D scaffold to differentiate induced pluripotent and embryonic stem cells (Levy et al., 2016). These approaches have also been studied in models and clinical trials for Alzheimer's, Huntington's, and Amyotrophic lateral sclerosis (Tronci et al., 2015) (Bishehsari et al., 2016; Tanna and Sachan, 2014; Waldau and Shetty, 2008).

Although clinical trials found robust, lasting symptomatic effects using human fetal ventral mesencephalic tissue transplants, numerous concerns were raised such as heterogeneity of outcome and graft-induced dyskinesia (Politis and Lindvall, 2012). Genomic instability and risk of tumor formation are other disadvantages when it comes to using embryonic stem cells (Goodarzi et al., 2015). Magnetic or fluorescence-based sorting techniques and directed differentiation may be used to reduce the number of tumorigenic embryonic stem cells from fetal tissue grafts, but tumor formation is still a primary concern, preventing FDA-approval of human ESC derived cells for transplants in PD patients (Brederlau et al., 2006; Lindvall, 2016; Shen et al., 2016). Fetal brain neural stem cells have a lower risk of tumor formation and immune rejection and are able to differentiate into dopaminergic neurons, however ethical issues and tissue availability substantially contribute

to reduced feasibility of using such grafts, despite the demonstrated graft survival and significant reduction in motor deficits resulting from human fetal tissue transplantation in PD patients (Goodarzi et al., 2015; Shen et al., 2016). Another concern is that α-synuclein actually gets transferred into the grafts, reducing survival due to prion like propagation of the misfolded protein (Angot et al., 2012; Lindvall, 2016; Narkiewicz et al., 2014). It should be noted that nonmotor symptoms are not alleviated by implantation and may even be worsened; however, improvement in altered neurogenesis and recruitment of endogenous neural stem cells may provide some relief from both motor and nonmotor functions (Lamm et al., 2014; Lindvall, 2016) .

Thus, a focus on dopaminergic neuroblasts derived from other stem cell tissue sources has emerged. Induced pluripotent stem cells (iPSCs) derived from fibroblasts and bone marrow-derived mesenchymal stem cells (MSCs) are a more reliable and realistic source of stem cells by avoiding both ethical concerns and immunologic consequences (Ambasudhan et al., 2014; El-Sadik, 2010; Sanchez-Danes et al., 2012). With iPSCs, there is still some concern of tumor formation (Sanchez-Danes et al., 2012). MSCs have been shown to modestly improve motor performance in mice yet have not resulted in adverse effects in humans (Goodarzi et al., 2015). Other sources of allograft tissue should be further explored, such as sertoli cells which have been shown to be protective in PD models due to their trophic effects and immunosuppressive properties (Liu et al., 1999; Sanberg et al., 1997; Willing et al., 1999). These cells could first be reprogrammed to overexpress neurotrophic factors or other signaling factors to aid in both dopaminergic neuronal differentiation and survival of the grafted tissue (Chen, 2015; El-Sadik, 2010; Levy et al., 2016; Li et al., 2013a).

To cause non-mesencephalic NSCs to differentiate into dopaminergic neurons, groups have mimicked embryonic development through activating factors such as sonic hedgehog (Shh), fibroblast growth factor 8 (fgf8), and Wnt1 to result in a series of downstream effects (Bonzano et al., 2016; Bovetti et al., 2013; Luo and Huang, 2016; Rossler et al., 2010; Wenker et al., 2016). This lead to Pitx3 expressing neurons but only a small number of cells expressed TH (Rossler et al., 2010). One study showed that human neural stem cells transplanted into an MPTP primate model showed glial lineage (Bjugstad et al., 2008). Other studies suggest that overexpression of Oct4, Nurr1, or GDNF may be required for adult NPC to dopaminergic cell differentiation *in vivo* (Cave et al., 2014; Deleidi et al., 2011; Ding et al., 2016; Dong et al., 2016; Sakthiswary and Raymond, 2012; Shim et al., 2007; Wakeman et al., 2014).

In culture, these mechanisms are not fully defined yet, making it clear that neighboring cells at the graft site play a major role in dopaminergic neuronal differentiation. For example, Nurr1 and glial-derived factors were found to be essential to grafts producing dopamine (Wagner et al., 1999). Histone deacetylases are a potential way of encouraging stem cells to differentiate towards neural progenitor cells (Yang et al., 2014). Neurotrophic effects due to diet and exercise could also benefit patients by increasing neurogenesis (Monteiro-Junior et al., 2015; Shah and Duda, 2015). Cell confluency, three dimensional structure, and injury-induced factors also positively affects survival and expression of TH, suggesting possible feed forward mechanisms between neurons (Ding et al., 2016; Ni et al., 2013; Singh et al., 2016; Yang et al., 2014). Judging by the robust effects of PK2 on other cell types as demonstrated in our recent publications (Gordon et al., 2016), could PK2 be one

of these “help me” injury factors necessary for recruitment or dopaminergic differentiation of stem cells?

Gene therapy

A number of studies have overexpressed neuroprotective proteins in the nigrostriatal tract using adeno-associated virus (AAV) for gene therapy in animal models. AAVs do not replicate and rarely integrate into the host genome (Albert et al., 2017; Kaplitt et al., 1994). It is considered a safe viral vector for gene delivery in human disease, and is able to transduce many different neuronal cell types depending on the combination of serotypes and promotors (Albert et al., 2017; Kaplitt et al., 1994). In the rodent brain and clinical trials, AAV serotype-2 (AAV2) is most commonly studied (Burger et al., 2004; Cearley et al., 2008). AAV9 has received some attention for its capability of crossing the blood-brain-barrier to some degree (Foust et al., 2009). However, evidence suggests that AAV5 may be more efficient at transducing neurons (Albert et al., 2017; Shevtsova et al., 2005). One limitation of this approach is that only a small amount of DNA can be inserted (Albert et al., 2017; Lim et al., 2010).

Several clinical trials applying AAV-based gene therapy approaches are currently underway and are summarized in Table 1 (Albert et al., 2017; Kirik et al., 2017) (Valdes and Schneider, 2016). Neurotrophic factors are a popular disease modifying strategy for PD research, with glial cell line-derived neurotrophic factor (GDNF) currently being the most studied in rodent, non-human primate studies, and clinical trials (Airaksinen and Saarma, 2002; Bjorklund et al., 2000; Johnston et al., 2009; Kells et al., 2010; Kirik et al., 2000;

Wang et al., 2002). Ongoing studies from our lab suggest that PK2 may increase GDNF expression (Neal, 2016).

Table 3. *Gene therapy clinical trials currently underway. From Blits and Petry et al., 2016.*

Product	Highest dose (GC total)	Volume (µl)	Target	Mode of action	Sponsor	Indication for efficacy in Phase I safety studies
AAV-2-GAD	1E12	50	Subthalamic nucleus (STN)	GABA-mediated inhibition to affected areas	Neurologix	Modest UPDRS improvement
AAV-2-AADC	0.3E12	50-450	Putamen	Replacement missing AADC gene	Genzyme/Voyager	Modest UPDRS improvement Increased FluoroDOPA uptake
AAV-NTN	0.5E12	50	Putamen	Trophic support	Ceregene	No improvement UPDRS
AAV-NTN	2.4E12	50 + 10	Putamen + Substantia Nigra	Trophic support	Ceregene	Modest UPDRS, especially in early diagnosed patients
LV-TH-GCH-AADC	1E8 TU*	50	Putamen	Internal dopamine synthesis	Oxford Biomedica	Modest UPDRS improvement
AAV-2-GDNF	3E12	450	Putamen	Trophic support	UCSF/NIH	Not yet disclosed

Prokineticin-2

Prokineticin-2 is a secreted AVIT family peptide with two GPCR receptors, PKR1 and PKR2 (Li et al., 2001; Negri et al., 2007; Ngan and Tam, 2008). As the mammalian homologues to MIT-1 and Bv8, Prokineticin-1 (PK1) and PK2 proteins were first discovered as potent media tors of gastrointestinal motility found within in the black mamba snake venom and toad skin (Li et al., 2001). Prokineticins contain distinctive structural motifs including a N-terminal AVITGA sequence and ten cysteine residues which form disulfide bonds in the C-terminal domain, both of which are essential for receptor binding and activity (Bullock et al., 2004; Maldonado-Perez et al., 2007; Monnier and Samson, 2008). Although PK1 does not have any known splice variants, PK2 can normally be found as the 81aa peptide or as the 102aa long, basic form of the peptide in the testis (Jilek et al., 2000; Maldonado-Perez et al., 2007).

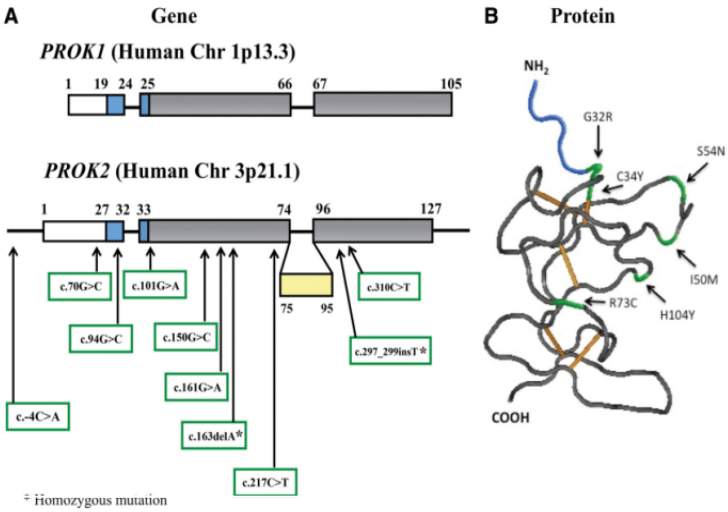


Figure 9.*Prokineticin-2 structure. From Maldonado-Perez et al.2007.*

Prokineticins and their receptors are differentially expressed throughout the mammalian body and utilize a variety of downstream signaling cascades, giving rise to a diverse array of physiological functions including angiogenesis, hematopoiesis, circadian rhythms, nociception, neurogenesis, chemotaxis, reproduction, and immune response (Alfaidy et al., 2014; Balasubramanian et al., 2011; Li et al., 2012a; Monnier and Samson,

2008; Negri and Lattanzi, 2012; Ng et al., 2005; Ngan and Tam, 2008). PK1 expression is widely found in steroidogenic organs and the gastrointestinal tract, while PK2 is distributed in the brain, blood and reproductive organs (Balasubramanian et al., 2011; Ferrara et al., 2004; Heck et al., 2015; Li et al., 2001; Zhou and Cheng, 2005). In the brain, PK2 is known to regulate olfactory bulb neurogenesis, by serving as a chemoattractant and detachment signal for NPCs in the SVZ and OB, respectively (Ng et al., 2005). Additionally, PK2 plays a role in circadian rhythms and food intake in the hypothalamus and migration of gonadotropin-releasing hormone neurons from the nose to the brain during development (Beale et al., 2013; Forni and Wray, 2015; Zhou and Cheng, 2005). Basic helix-loop-helix transcription factors, such as MASH1 and CLOCK, or hypoxia can regulate PK2 expression in these distinct biological processes (Zhang et al., 2007; Zhou, 2006).

Pathological implications resulting from overexpression of PK2 involve tumor angiogenesis, aneurism, ischemia, neuropathic pain and inflammation, whereas knockout studies show that deficits cause Kallman syndrome, a disease marked by reproductive and olfactory problems associated with atrophy in the corresponding regions (Abou-Hamdan et al., 2015; Cheng et al., 2012; Choke et al., 2009; Kurebayashi et al., 2015; Lattanzi et al., 2015; Martin et al., 2011; Matsumoto et al., 2006; Wang et al., 2016; Watson et al., 2012). In Chapter 5, we explore the role of PK2 in altered neurogenesis in PD models.

CHAPTER 2:

CHARACTERIZATION OF NONMOTOR SYMPTOMS IN THE MITOPARK MOUSE MODEL OF PARKINSON'S DISEASE

A manuscript to be submitted to *Brain*

Langley, Monica R; Ghaisas, Shivani; Ay, Muhammet; Jin, Huajun;

Anantharam, Vellareddy; Kanthasamy, Arthi; Kanthasamy, Anumantha G

[1]Parkinson Disorders Research Program, Iowa Center for Advanced Neurotoxicology, Department of Biomedical Sciences, Iowa State University, Ames, IA 50011.

Address correspondence and proof requests to

Dr. A.G. Kanthasamy, Parkinson Disorders Research Laboratory, Department of Biomedical Sciences, 2062 CVM Building, Iowa State University, Ames, IA, 50011

Tel.: (515) 294-2516, Fax: (515) 294 2315, E-mail: akanthas@iastate.edu

Running title: Nonmotor Symptoms in MitoPark Mice

Keywords: Parkinson's disease, behavior, nonmotor, MitoPark

Abstract

Mitochondrial dysfunction has been implicated as a key player in the pathogenesis of Parkinson's disease. The MitoPark mouse, a transgenic mitochondrial impairment model recently developed by specific inactivation of TFAM in dopaminergic neurons, spontaneously exhibits progressive motor deficits and neurodegeneration, recapitulating several features of PD. Since nonmotor symptoms are now recognized as important features of the prodromal stage of Parkinson's, we monitored the clinically relevant motor and nonmotor symptoms from ages 8-24 wks in MitoPark mice and their littermate controls. As expected, motor deficits in MitoPark mice begin around 12 wks and become severe by 16-24 wks. Interestingly, male MitoPark mice showed spatial memory deficits before female mice, beginning at 8 wks and becoming most severe at 16 wks, as determined by Morris water maze. When compared to age-matched control mice, MitoPark mice exhibited olfactory deficits in novel and social scent tests as early as 10-12 wks. MitoPark mice between 16-24 wks spent more time immobile in forced swim and tail suspension tests, and made fewer entries into open arms of the elevated plus maze, indicating a depressive- and anxiety-like phenotype, respectively. Importantly, depressive behavior as determined by immobility in forced swim test was reversible by antidepressant treatment with desipramine. Collectively, our results indicate that MitoPark mice progressively exhibit deficits in cognitive learning and memory, olfactory discrimination, and anxiety- and depressive-like behaviors. Thus, MitoPark mice can serve as an invaluable model for studying motor and nonmotor symptoms in addition to studying pathology in Parkinson's disease.

Introduction

Parkinson's disease is a chronic, progressive neurodegenerative disorder affecting about 5 million people worldwide. Parkinson's disease neuropathology is characterized by a loss of dopaminergic neurons in the substantia nigra (SN) of the brain, leading to a functional loss of dopamine in the striatum and severe motor deficits. Additionally, accumulations of abnormal alpha-synuclein (α-syn) protein form Lewy bodies and Lewy neurites, hallmarks of Parkinson's disease pathology. Several genes associated with Parkinson's disease include PINK1, Parkin, DJ-1, and LRRK2; however, the vast majority of cases are considered idiopathic, implicating environmental factors such as metals, pesticides, and other toxins in the etiology of the disease. Cardinal motor symptoms such as bradykinesia, tremor, rigidity, and postural instability are still classically used for the clinical diagnosis of Parkinson's disease. Neuroinflammation, oxidative stress and mitochondrial dysfunction are thought to contribute to the pathogenesis of Parkinson's disease (Varcin et al., 2012, Subramaniam and Chesselet, 2013, Kanthasamy et al., 2010). Current therapies, including levodopa (L-DOPA), monoamine oxidase inhibitors, and dopamine agonists, treat the symptoms yet ultimately cannot interrupt or slow down the neurodegenerative process. Furthermore, most commonly prescribed treatments do not address the full scope of symptomology in Parkinson's disease patients.

In addition to the characteristic motor symptoms, nonmotor symptoms such as hyposmia, sleep disturbances, gastrointestinal (GI) dysfunction, autonomic and cognitive deficits negatively affect quality of life and cost of living for Parkinson's disease patients. Although often overlooked, nonmotor symptoms are a frequent cause of hospitalization and diminished quality of life for Parkinson's disease patients (Chaudhuri and Schapira, 2009).

Neuropsychiatric symptoms in Parkinson's disease include depression, dementia, anxiety, apathy, and cognitive dysfunction. Olfactory deficits are observed in more than 95% of those affected by Parkinson's disease, and depression is estimated to affect more than one-third of Parkinson's disease patients (Haehner et al., 2011, Meyer et al., 2014, Chaudhuri and Schapira, 2009). Interestingly, a recent cross-sectional observational study found that nonmotor deficits precede motor symptoms in later-onset PD, while younger patients showed the opposite trend (Zhang et al., 2016). Although behavioral tests are available to study various nonmotor symptoms in rodent species, large data gaps still remain in understanding the nonmotor phenotype of many toxin-based and genetic models of Parkinson's (Taylor et al., 2010).

In many toxin-based models of PD, rodent species either do not suffer from PD-related nonmotor symptomology or a complete behavioral phenotyping has not yet been performed. For example, intraperitoneally injected MPTP-treated animals do not display any olfactory impairment, although intranasal MPTP can functionally damage the olfactory epithelium (Kurtenbach et al., 2013). Although gastric emptying and small intestine transit are unaffected by MPTP, the loss of enteric dopaminergic neurons due to the treatment increases colon motility (Anderson et al., 2007). Paraquat or paraquat/maneb co-administration in rodent models has only been shown to induce anxiety- and depressive-like behaviors (Litteljohn et al., 2009, Tinakoua et al., 2015, Campos et al., 2013). Interestingly, a recent study revealed that rotenone-treated zebrafish display motor, olfactory, and neuropsychiatric symptoms (Wang et al., 2017).

In transgenic mouse models, only a few α-syn mutant animal models have been shown to have olfactory and GI functional changes (Fleming et al., 2008, Wang et al., 2008, Dawson et al., 2010). Parkin knockout mice have spatial memory impairments, but do not show evidence of olfactory dysfunction, anxiety, depression, or motor deficits (Rial et al., 2014). One group has recently identified olfactory dysfunction in the LRRK2 mouse model (Dranka et al., 2014). The presence of nonmotor symptoms in PD models would be particularly useful if they can be characterized as early onset and progressive similar to clinical Parkinson's. Future studies identifying the potential nonmotor abnormalities in each model could be utilized in the development of therapies to mediate nonmotor symptoms and also to screen for adverse effects resulting from dopaminergic therapies.

Recapitulating many of the hallmark features of Parkinson's Disease, the MitoPark mouse was created by inactivation of mitochondrial transcription factor A (TFAM) in the nigrostriatal pathway through control of the DAT promoter. MitoPark mice exhibit adult-onset progressive dopaminergic neurodegeneration, aggregation of protein in nigral tissues, and L-dopa responsive motor deficits (Ekstrand et al., 2007, Ekstrand and Galter, 2009). Furthermore, MitoPark mice were recently discovered to display certain nonmotor symptoms such as all-light- or all-dark-induced circadian rhythm dysfunction and early cognitive deficits (Fifel and Cooper, 2014, Li et al., 2013). In the present study, we systematically characterized the nonmotor behavioral phenotype of Parkinson's disease in the MitoPark mouse model. The overarching hypothesis is that MitoPark mice will also display characteristic nonmotor symptoms of Parkinson's disease.

Materials and Methods

Chemicals

Dopamine hydrochloride, 3-4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) were all purchased from Sigma (St Louis, MO). Halt protease and phosphatase inhibitor cocktail was obtained from Thermo Fisher (Waltham, MA). Bradford assay reagent and Western blotting buffers were purchased from Bio-Rad (Hercules, CA). Anti-4-hydroxynonenal antibody was purchased from R&D Systems (Minneapolis, MN, (Ghosh et al., 2016)). CREB and p-CREB antibodies were obtained from Cell signaling (Boston, MA, (Jin et al., 2011)). The anti-mouse and anti-rabbit secondary antibodies (Alexa Fluor 680 conjugated anti-mouse IgG and IRdye 800 conjugated anti-rabbit IgG) were purchased from Invitrogen and Rockland Inc., respectively.

Experimental design

Experimenter was blinded to mouse genotype for each behavioral test by flipping of cage cards and randomizing order prior to beginning the experiment. A third party was used to conceal treatment and vehicle solution identity prior to drug administration. Genotype and administration blinding was decoded after data acquisition was complete. Power analysis using the “fpower” function in SAS was used to determine the following animal requirements based on 80% power, alpha=0.05, and four groups. Clinically significant differences and standard deviations used to determine delta were taken from ANOVA analyses of previous studies in our lab. Based on preliminary forced swim test data, a minimum of 11 animals per group will be used for behavioral animal studies to detect an effect size of 1.5.

Animal treatment

MitoPark mice were originally kindly provided and generated by Dr. Nils-Goran Larson at the Karolinska Institute in Stockholm (Ekstrand et al., 2007). All mice for this study were bred, maintained, genotyped, and further characterized at ISU. MitoPark mice (DAT +/Cre, TFAM LoxP/LoxP) and their littermate controls (DAT+/+, TFAM +/LoxP) were fed *ad libitum* and housed in standard conditions approved and supervised by the Institutional Animal Care and Use Committee (IACUC) at ISU. Mice were weighed and subjected to behavioral tests every two weeks. Neurochemical, biochemical, and histological studies were performed following sacrifice at age 24 wks.

Motor function test

For open-field test, a VersaMax system (VersaMax monitor, model RXYZCM-16, and analyzer, model VMAUSB, AccuScan, Columbus, OH) was used for monitoring locomotor activity. For horizontal and vertical activity and corresponding plots, mice were acclimated for 2 min prior to recording for 10 min using the VersaMax system. RotaRod equipment (AccuScan) was used to test coordination of movement as previously described (Ghosh et al., 2012). Briefly, time spent on rod rotating at 20 rpm was measured for a maximum of either 20 min or five trials, each of which ended with a mouse falling from the rod.

Neuromuscular function and muscular strength

Each mouse was lifted over the GSM's baseplate by the tail so that its forepaws could grasp onto the steel grip. Each mouse was then gently pulled backward by the tail until its

grip released. The GSM measures the maximal force before the mouse releases the bar (Danilov and Steward, 2015). Three trials were performed for each mouse with a 1-minute resting period between trials. Latency to release (sec) and gram-force (gF) were recorded.

Social discrimination and novel scent tests

To determine the olfactory function of control and MitoPark mice, we used a social discrimination test as previously described (Ngwa et al., 2014). However, this procedure was adapted to use AnyMaze tracking software (AMS, Stoelting Co., Wood Dale, IL) to determine time spent sniffing based on the animals head being within a defined zone (1-cm perimeter around dish) surrounding the bedding. Total time spent sniffing the opposite sex's bedding (from a group-housed cage) was recording during a 3-min trial. Similarly, AMS was used to determine time sniffing a novel scent as described by others (Taylor et al., 2009). During a 3-min trial, time spent sniffing scented and non-scented zones was recorded using AMS. Scents used were lemon, peppermint and vanilla, whereas water served as the non-scent.

Cognitive testing

A six-day Morris water maze (MWM) protocol was used as described previously in an Alzheimer's disease mouse model (Bromley-Brits et al., 2011). Briefly, each mouse gets five 1-min trials per day. On the first day, the platform is visible and its position changes between trials to show that the ability to see and swim to the platform is not impaired by visual or motor deficits. On days 2-5, mice are placed into the MWM tank filled with white, opaque water to learn to find a hidden platform whose position does not change between trials. The time required for a mouse to find and mount the platform is reported here as

escape latency. Finally, the platform is removed on the sixth day to show memory retention of the previously located platform, reported here as time spent searching in the quadrant that contained the platform during Days 2-5. Each trial was monitored using AMS. Each inter-trial interval was a minimum of 20 min, during which time mice were warmed and dried on heating pads placed under cages. Water temperature in the MWM tank was maintained at 23±1°C.

Forced swim and tail suspension tests

For depressive phenotyping, tail suspension and forced swim tests were used. During tail suspension trials, mice were individually suspended at a height of 30 cm by attaching the tail to a horizontal ring stand bar using adhesive tape. Each 6-min test session was videotaped and scored using AMS for escape-oriented behavior/mobility and bouts of immobility. The time spent immobile was recorded for each mouse as a correlate of depressive-like behavior (Taylor et al., 2010, Can et al., 2012b).

For the Porsolt "forced swim" test, mice were placed individually in glass cylinders (24 × 16 cm) with 15 cm of water maintained at 25°C as previously described (Porsolt et al., 1979, Can et al., 2012a). Mice were left in the cylinder and their behavior was videotaped from the side of the cylinder for 6 min. After the first 2 min, the total duration of time spent immobile was recorded during a 4-min test. A mouse was deemed immobile when it was floating 65% passively for at least 2.5 sec according to AMS.

Elevated plus maze

Time spent in the open arms is inversely correlated with an anxious phenotype (Lister, 1987). Mice were placed into the center of the elevated plus maze (Stoelting) and

video-recorded for 10 min as previously described (Komada et al., 2008). Time spent in open arms was determined by AMS.

Sleep latency test

Animals were allowed to acclimate 4 hours before being awakened by gentle handling. Latency to sleep was determined by observer monitoring behavioral signs of sleep. Sleep was defined as 2 min of uninterrupted sleep behavior and 75% of the next ten minutes spent in sleep behavior as previously described (Taylor et al., 2009).

High Performance Liquid Chromatography

Striatum, hippocampus, and olfactory bulb samples were prepared and processed for HPLC as described previously (Gordon et al., 2016). Briefly, dissected brain regions were placed in a buffer comprising 0.2 M perchloric acid, 0.05% Na2EDTA, 0.1% Na2S2O5 and isoproterenol (internal standard) to extract monoamine neurotransmitters. Monoamine lysates were placed in a refrigerated automatic sampler (model WPS-3000TSL) until being separated isocratically by a reversed-phase C18 column with a flow rate of 0.6 ml/ min using a Dionex Ultimate 3000 HPLC system (pump ISO-3100SD, Thermo Scientific, Bannockburn, IL). Electrochemical detection was achieved using a CoulArray model 5600A coupled with an analytical cell (microdialysis cell 5014B) and a guard cell (model 5020) with cell potentials set at -350, 0, 150, and 220 mV. Data acquisition and analysis were performed using Chromeleon 7 and ESA CoulArray 3.10 HPLC Software and quantified data were normalized to wet tissue weight.

Western blot

Protein lysates from the striatum and substantia nigra were prepared in RIPA buffer with protease and phosphatase inhibitors and ran on a 12-15% SDS-PAGE as previously described (Jin et al., 2014) before being transferred to a nitrocellulose membrane. After blocking for 1 h, membranes were incubated with primary antibodies at 4ºC overnight. Next, membranes were incubated with secondary antibodies (Alexa Fluor 680 and Rockland IR800) at RT for one hour and images were captured via Licor Odyssey imager. Densitometric analysis was done using ImageJ software.

Statistical analysis

All behavioral tests were analyzed as repeated measures two-way ANOVA with Bonferroni post-tests. Biochemical and neurochemical analyses were performed by two-tailed student's t-test. For ANOVA and t-tests, alpha level of $p \leq 0.05$ was used to determine significance.

Results

Progressive Motor Deficits in MitoPark Mice

Previous studies have characterized motor deficits in MitoPark mice (Ekstrand et al., 2007). In our laboratory, we have found that some female mice exhibit poor condition after age 24 wks. Therefore, we decided to sacrifice at 24 wks instead of the previously reported 40 wks (Ekstrand and Galter, 2009, Ekstrand et al., 2007). As expected, male and female MitoPark mice revealed decreased horizontal and vertical activities which progressively worsened over time, beginning at 12-14 wks of age (Fig. 1A-C). MitoPark mice also spent significantly less time on the RotaRod from 12 wks onward (Fig. 1D). No significant changes were observed for grip strength at any age between MitoPark mice and littermate controls (Fig. 1E), indicating forelimb neuromuscular function remained intact. Similar to previous reports, MitoPark mice showed a significant reduction in body weight at 20 wks for males and at 22 wks for females (Fig. 1F, Supplementary Fig 1). Male and female data were significantly different for certain behavioral parameters and were therefore plotted separately as well (Supplementary Fig 1).

Cognitive dysfunction in MitoPark Mice

To screen for cognitive deficits associated with spatial learning and memory, we next applied a six-day MWM protocol as described in Fig. 2A and depicted at 24 wks in Fig. 2B. Days 1-5 are track plots of the animals path to the visible (day1) or hidden (days 2-5) platform. Day 6 occupancy plots reveal time spent in each location during a one minute retention trial with the platform removed. Recently, a group has shown that cognitive dysfunction precedes motor deficits in MitoPark mice using the Barnes Maze (Li et al.,

2013). Similarly, we report that 8-wk males exhibited impairments in the learning phase of the MWM (Fig. 2C-E). To our surprise, female MitoPark mice did not show an increase in escape latency until 12 wks of age (Fig. 2F-H). Recent studied have suggested that sex and age both impact spatial learning task performance, such as during the Morris water maze (Tropp Sneider et al., 2015). These variations in long term potentiation, neurogenesis, and spatial learning ability could potentially explain the differences seen in the development of cognitive deficits in this model. Other groups studying cognition in MitoPark mice did not comment on any sex differences in their learning and memory task. At 24 wks, both male and female MitoPark mice were unable to find the platform during the MWM learning phase (Fig. 2E, H). Deficits in the memory retention testing phase of MWM were apparent by 16 wks of age as depicted and quantified in Figures 2I and 2J, respectively. Overall, these results not only confirm previous findings that learning deficits precede motor dysfunction in the MitoPark mouse model, but also further describe advanced spatial memory problems after 16 wks of age and reveal sex differences in learning the MWM platform location.

Neuropsychiatric symptoms in MitoPark mice

Depression is estimated to affect more than half of Parkinson's patients and largely impacts patients' quality of life (Balestrino and Martinez-Martin, 2017). MitoPark mice were monitored every two wks for depressive and anxiety-like symptoms from 8-24 wks of age. The tail suspension test (TST) revealed depressive-like behavior as indicated by increased immobility time in MitoPark mice at 16 weeks when compared to age-matched littermate control mice (Fig 3A). Control mice also showed increased immobility during TST at 24 wks. During the Porsolt Forced Swim Test (FST), a significant increase in immobility

occurred from 14 wks onward in MitoPark mice, while control mice did not show any significant increase in immobility over time (Fig 3B).

To further support that this finding was due to behavioral despair and not motor dysfunction, we treated a subset of 16- and 24-wk mice with desipramine (5 mg/kg, i.p.), an antidepressant that increases neurogenesis, and performed the FST 30 min post-treatment. In accordance with other studies showing antidepressant efficacy through neurogenesis, our Western blotting revealed increased CREB phosphorylation in the hippocampus of FST-tested mice versus untested controls (Fig 3D, E). However, MitoPark mice did not show significant induction of pCREB unless treated with desipramine. Importantly, antidepressant treatment restored CREB phosphorylation to control levels (Fig 3C, D) and reduced immobility during the FST (Fig 3E). These data show that increasing pCREB in the hippocampus of MitoPark mice attenuated behavioral despair. Furthermore, the fact that immobility was reduced by antidepressant treatment suggests that motor dysfunction in the MitoPark model is not the cause of immobility observed during the FST.

We also performed a 10-min Elevated Plus Maze trial to test for anxiety-like behavior in MitoPark mice. The open arms are indicated by black arrows and closed arms by red arrows in Figure 3F. Here, we first show a significant decrease in the time spent in the open arms at 14 wks of age (Fig 3G), suggesting anxious behavior in MitoPark mice. Taken together, we have identified neuropsychiatric symptoms present starting from 14 wks in MitoPark mice, concurrent with the onset of motor deficits in this model.

Olfactory dysfunction in MitoPark mice

Changes in olfaction are highly prevalent in Parkinson's patients and may occur decades prior to onset of motor dysfunction, making screening of olfactory deficits a

potential prognostic tool in early Parkinson's disease (Ottaviano et al., 2016, Visanji and Marras, 2015). Representative occupancy plots from a 3-min trial of the social discrimination test (Fig 4A) and novel scent test (Fig 4B) at ages 8-24 wks reveal a reduced preference for the scented region (arrow) over time in MitoPark mice but not age-matched controls. Olfactory deficits, as indicated by significant differences in percent investigatory time during the social discrimination and novel scent tests, can be observed at 14 and 16 wks, respectively (Fig 4C, D). Also, a decrease in the number of entries into the scented region during the social discrimination test can be observed as early as 10 wks (Fig. 4E), while during the novel scent test, a significant reduction in entries began at 12 wks of age (Fig. 4F). These results indicate that olfactory deficits begin prior to the onset of motor symptoms in MitoPark mice.

Biochemical changes correlating with observed behavioral symptoms in MitoPark mice

Next, we tested for correlations between specific biochemical changes and the occurrence of key nonmotor symptoms. Studies have shown that CREB phosphorylation and BDNF levels increase in mice post MWM in a time-dependent manner (Lee et al., 2015, Min et al., 2015, Cho et al., 2013). Hence, we sacrificed mice 10-30 min after the last MWM retention trial and performed Western blotting for CREB, phospho-CREB, and BDNF protein levels. A significant reduction in CREB phosphorylation (Fig 5A, C) and BDNF (Fig 5A, D) protein levels in the hippocampus may be associated with the observed cognitive deficits.

Conversely, an increase in CREB phosphorylation and BDNF in the striatum may be related to the depressive phenotype observed (Fig 5B, F, and G). Within the mesolimbic

dopamine circuit, increased BDNF through CREB activation mediates susceptibility to stress (Krishnan and Nestler, 2008, Vaidya and Duman, 2001). In contrast, stress decreases hippocampal levels of BDNF and neurogenesis through CREB activity and cortisol concentrations (Krishnan and Nestler, 2008). Oxidative stress has also been implicated in a variety of neuropsychiatric disorders, with particularly strong evidence in depression and anxiety studies (Balmus et al., 2016, Ng et al., 2008). In both striatal and hippocampal tissues, we observed a significant increase in 4-HNE, a lipid peroxidation product that results from oxidative damage (Fig 5A, B, E, and H). Mitochondrial dysfunction is expected to lead to oxidative damage in neurodegenerative disorders (Islam, 2017), potentially representing the mechanism underlying the occurrence of neuropsychiatric symptoms in the MitoPark model.

Researchers have attempted to link olfactory dysfunction to α-syn deposition, since both occur early in Parkinson's pathogenesis (Reichmann et al., 2016). However, we did not see an increase in oligomeric protein in the olfactory bulb of MitoPark mice. Similar to what was reported in the substantia nigra of MitoPark mice (Ekstrand et al., 2007), we did see a significant increase in protein aggregation in striatal tissues as determined by slot blot for A11 anti-oligomeric protein antibody (Figure 5I, J).

Neurochemical changes in MitoPark mice

Depressive-like behaviors in toxin-based models of Parkinson's disease have been predominantly associated with reductions in hippocampal serotonin and striatal dopamine (Santiago et al., 2010). No significant changes were observed in hippocampal neurotransmitters (Fig 6A), indicating that observed changes are instead possibly due to neurogenesis or plasticity modifications. As anticipated, strong reductions in dopamine and

its metabolites were observed in the striatum of MitoPark mice, corresponding to the motor phenotype of the model (Figure 6B).

In our study, levels of dopamine and serotonin were reduced in the olfactory bulbs of 24-wk MitoPark mice (Figure 6C), which may help explain the anosmia in this model. However, one other study reported a dopamine reduction in the olfactory bulb that did not occur until a later age (Branch et al., 2016). Because DOPAC also increased, we believe enhanced dopamine turnover occurred in the olfactory bulbs of MitoPark mice. Similarly, we found increased dopamine turnover in the brainstem (Fig. S4). Oligomeric protein did not significantly increase in the olfactory bulb (Fig 5I, J), thus the anosmia most likely resulted from neurochemical changes rather than protein aggregation. Future studies should explore the role of specific olfactory receptors in this model at various stages to better elucidate the mechanism of anosmia in MitoPark mice.

Discussion:

We demonstrate that in addition to progressive motor deficits, nonmotor symptoms including olfactory dysfunction, learning and memory deficits, and neuropsychological problems are also evident in MitoPark mice. We also observed strong neurochemical and biochemical changes that correlated well with key nonmotor symptoms. For example, reduced levels of dopamine and serotonin in the olfactory bulb of 24-wk MitoPark mice may help explain the anosmia in this model (Ferrer et al., 2012, Taylor et al., 2009). Also, reductions in CREB phosphorylation and BDNF in the hippocampus may be associated with the observed cognitive deficits and decreased neurogenesis. Although animal models and post-mortem studies do indicate adult neurogenesis is affected in Parkinson's disease, the exact mechanism of the changes and the correlation with nonmotor symptoms has not been directly explored. Future studies should explore the role of adult neurogenesis in relation to nonmotor deficits in Parkinson's disease models.

Conversely, increases in CREB phosphorylation, BDNF, and oxidative damage marker 4-HNE in the striatum may be related to the depressive-like behavior observed. No significant changes in sleep latency were observed (Fig S2), although one recent paper found that MitoPark mice display circadian rhythm dysfunction following an all-light or all-dark cycle (Fifel and Cooper, 2014). The lack of changes in grip strength indicate that neuromuscular function is maintained even when locomotor and coordination deficits are present. Because constipation and other GI problems are associated with early stages of PD, further studies should be done to comprehensively characterize the GI changes in MitoPark mice. Based on MPTP studies resulting in loss of dopaminergic neurons in the enteric

nervous system, changes in colon motility should specifically be explored (Anderson et al., 2007).

Animal models and clinical Parkinson's disease studies have shown that reduced dopamine levels correlate with a reduced proliferation of cells in neurogenic regions of the brain, which is thought to contribute to the nonmotor symptoms observed in Parkinson's disease (Regensburger et al., 2014, Chiu et al., 2015, Kuipers et al., 2014). However, for most mouse models of Parkinson's disease either nonmotor symptoms do not emerge or else nonmotor performance was never characterized, presenting a narrow understanding of therapeutic potential with current genetic and toxin-based models (Taylor et al., 2010). Interestingly, mice deficient in monoamine storage capacity display a progressive loss of dopaminergic cells in the SN, loss of striatal dopamine, motor deficits, α-syn accumulation and key nonmotor deficits (Taylor et al., 2009). Neuroinflammation is another mechanism implicated in the pathophysiology of Parkinson's disease and nonmotor symptoms, given that inflammatory cytokine levels have been positively correlated with nonmotor deficits in a number of recent studies (Menza et al., 2010, Lindqvist et al., 2012).

Many nonmotor deficits closely correlate with Lewy body deposition and begin in the prodromal stage of Parkinson's disease, prior to the classical motor deficits used for clinical diagnosis (Shulman et al., 2001, Schneider and Obeso, 2015, Pellicano et al., 2007). First, GI and olfactory disturbances are observed while α-syn pathology is evident in the olfactory bulb and the motor nucleus of the vagus nerve, which provides parasympathetic innervation to the GI tract (Burke et al., 2008). Next, synucleopathy in the hypothalamus, locus coeruleus, and raphae nucleus correlates with sleep disorders and neuropsychiatric symptoms such as depression and anxiety. Motor symptoms and clinical diagnosis then begin during

Braak stages 3-4, when the midbrain becomes involved. Finally, cognitive decline and dementia are found in association with cortical deposition of α-syn, similar to what is seen in dementia with Lewy bodies (Jellinger, 2009). In addition to protein aggregate deposition, alterations in adult neurogenesis and neurochemical changes are implicated in the development of nonmotor symptoms in Parkinson's disease (Schlachetzki et al., 2016, Lamm et al., 2014). Recent studies suggest that dopamine depletion and changes in α-syn may even synergistically contribute to the altered neurogenesis associated with nonmotor deficits (Schlachetzki et al., 2016).

Recent studies from our lab indicate that oxidative stress and microglia activation contribute to the neurodegenerative process in this model, as in human Parkinson's disease (ARS MP mito-apo paper). Our cognitive function data are supported by a recent study showing spatial learning and memory deficits in the Barnes maze and object recognition deficits that preceded the appearance of motor deficits in these animals (Fifel and Cooper, 2014, Li et al., 2013). However, our study expanded their findings by showing various ages of mice progressively worsening.

Collectively, our study demonstrates that in addition to progressive motor deficits, MitoPark mice also exhibit nonmotor symptoms including olfactory dysfunction, learning and memory deficits, and neuropsychological problems. The presence of these symptoms in combination with the progressive motor dysfunction makes the MitoPark mouse model of PD particularly valuable for mechanistic and drug discovery studies.

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Abbreviations

Alpha-synuclein (α-syn), AnyMaze software (AMS), Analysis of variance (ANOVA), cAMP response element-binding protein (CREB), dentate gyrus (DG), dopamine transporter (DAT), 3-4-dihydroxyphenylacetic acid (DOPAC), forced swim test (FST), gastrointestinal (GI), grip strength meter (GSM), high performance liquid chromatography (HPLC), homovanillic acid (HVA), immunohistochemistry (IHC), Iowa State University (ISU), leucine-rich repeat kinase 2 (LRRK2), levodopa (L-DOPA1), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), Morris water maze (MWM), olfactory bulb (OB), 6-hydroxydopamine (6-OHDA), Parkinson's disease (PD), PTEN-induced putative kinase 1 (PINK1), quantitative polymerase chain reaction (qPCR), rostral migratory stream (RMS), substantia nigra (SN), subventricular zone (SVZ), subgranular zone (SGZ), striatum (STR), tail suspension test (TST), mitochondrial transcription factor A (TFAM)

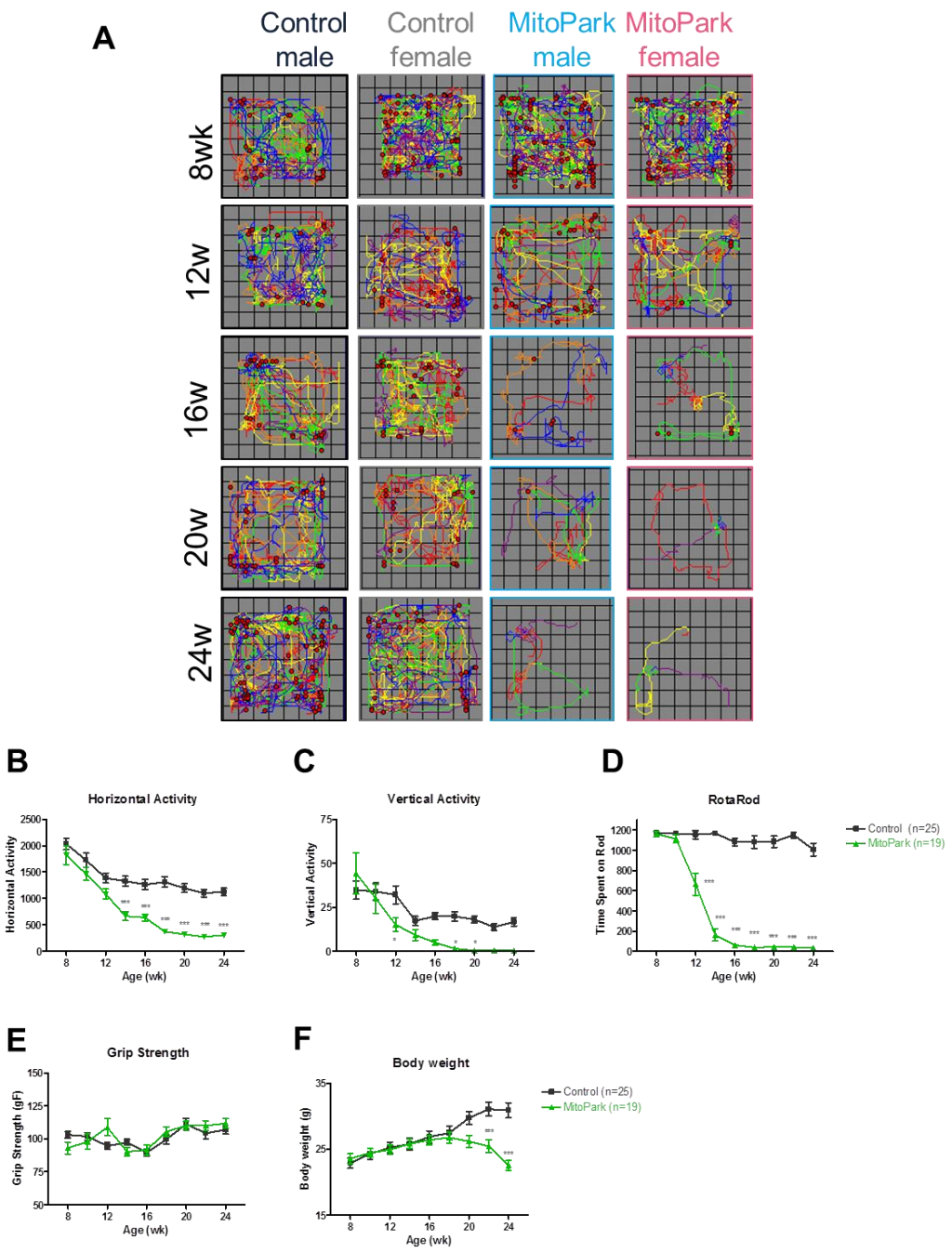

Figure 1. Progressive Motor Deficits in MitoPark Mice. *A*, VersaPlots from VersaMax open field test showing horizontal (lines) and vertical (red dots) motion during a ten minute testing interval. Quantification of horizontal (*B*) and vertical (*C*) activities as determined by

VersaMax analyzer during ten minute open field test. Average time spent on RotaRod at 20rpm during 5 trials. Grip strength (*E*) and body weights (*F*) from 8- to 24-wks of age in littermate control and MitoPark mice.

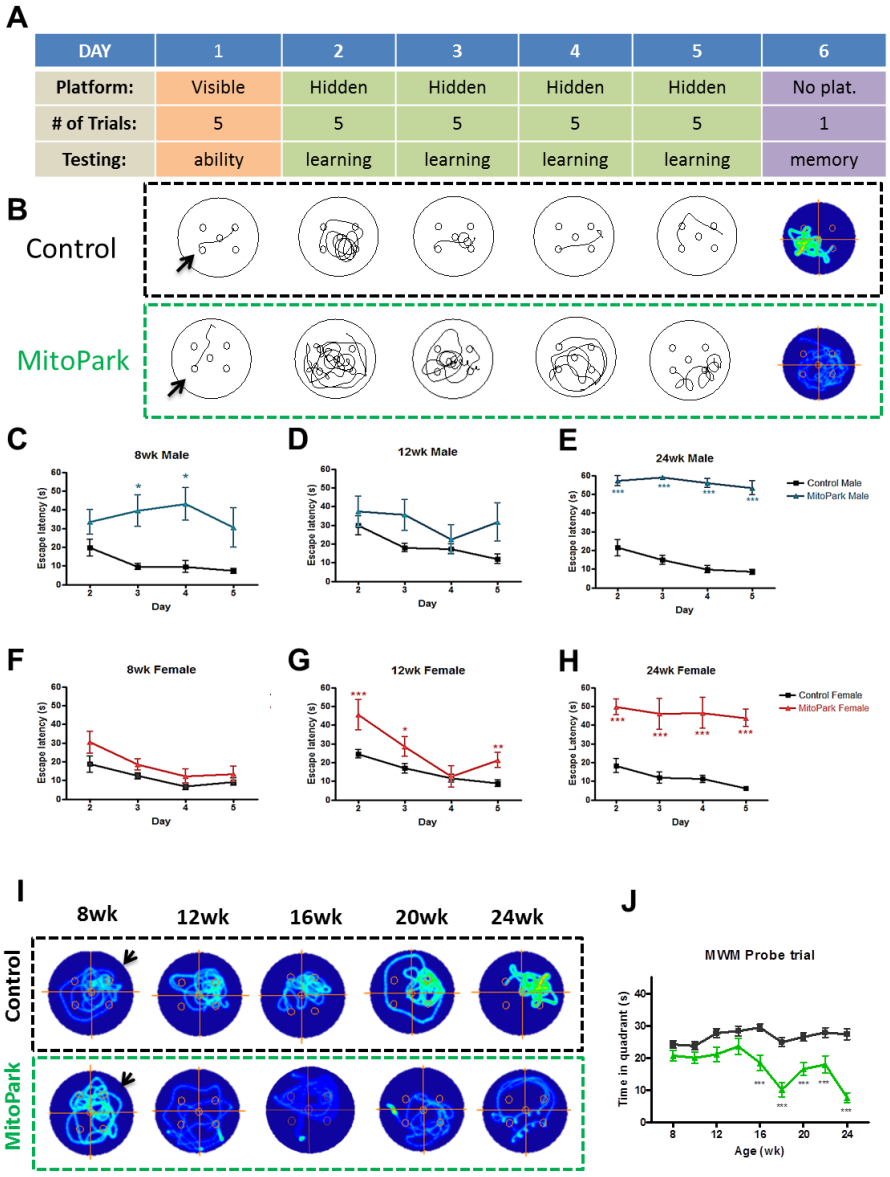

Figure 2. Cognitive dysfunction in MitoPark Mice. Schematic describing six day Morris water maze (MWM) protocol (*A*). Track plots from learning and occupancy plots from retention trials of MWM for littermate control and MitoPark mice (*B*). 8- (*C*), 12- (*D*), and 24-wk (*E*) MWM learning period for male mice. 8- (*F*), 12- (*G*), and 24-wk (*H*) MWM learning period for male mice. Occupancy plots (*I*) of mice during retention trial with arrow indicating the previous platform location and time in that quadrant (*J*).

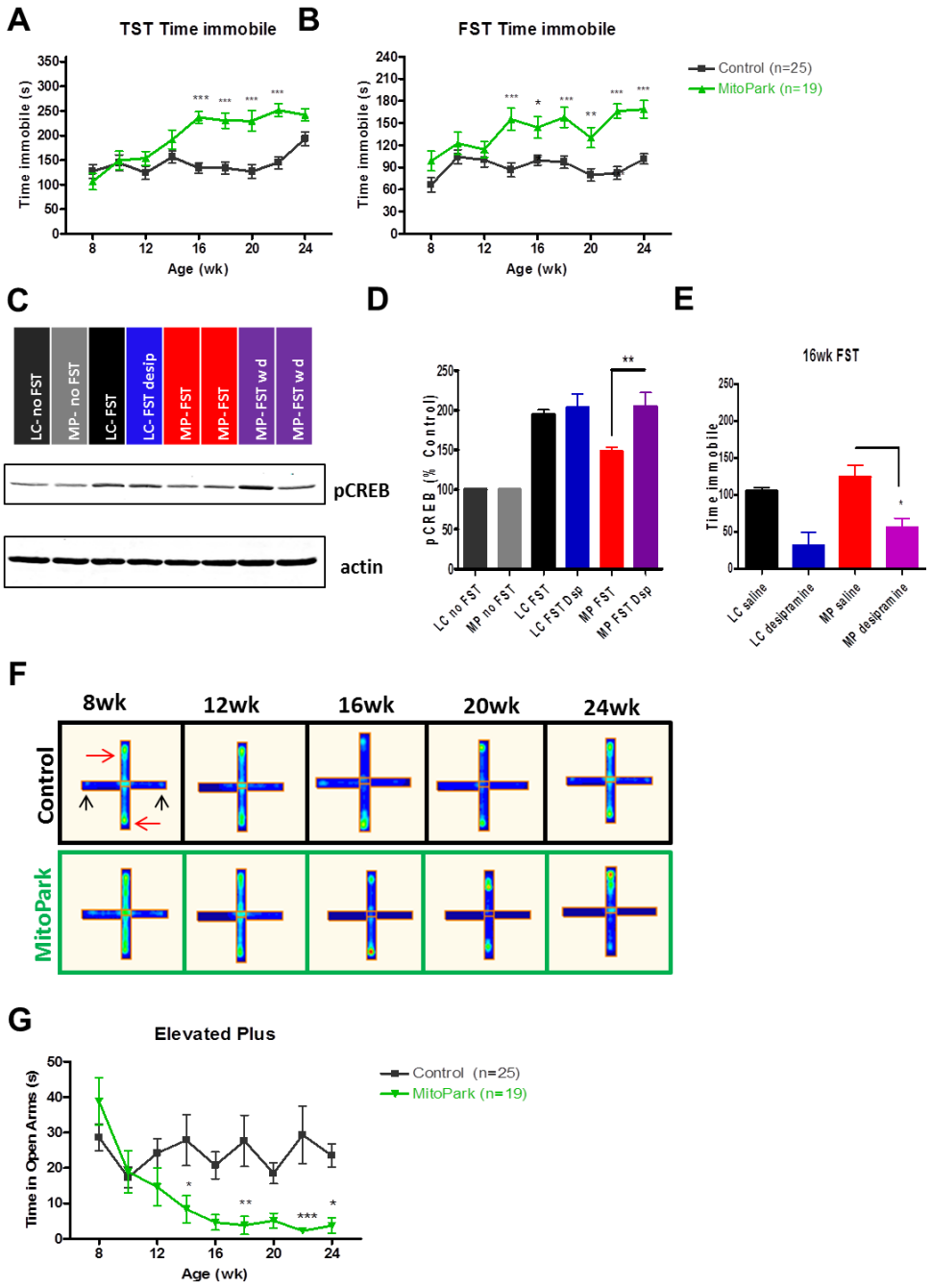

Figure 3. Neuropsychiatric symptoms in MitoPark mice. MitoPark mice were monitored biweekly for neuropsychiatric deficits from 8-24 weeks of age. The tail suspension test (TST) (*A*) and forced swim test (FST) (*B*) reveal depressive behavior in MitoPark mice at 16 and 14 weeks, respectively, when compared to age-matched control mice. Western blotting (*C*,

D) reveals increased CREB phosphorylation in FST-mice versus untested controls. *E*, Desipramine treatment (5mg/kg, i.p., 30 min prior to FST) significantly reduces immobility time during the FST in MitoPark mice. Occupancy plots (*F)* of closed (red arrow) and open (black arrow) arms of elevated plus maze and corresponding time in open arms (*G*).

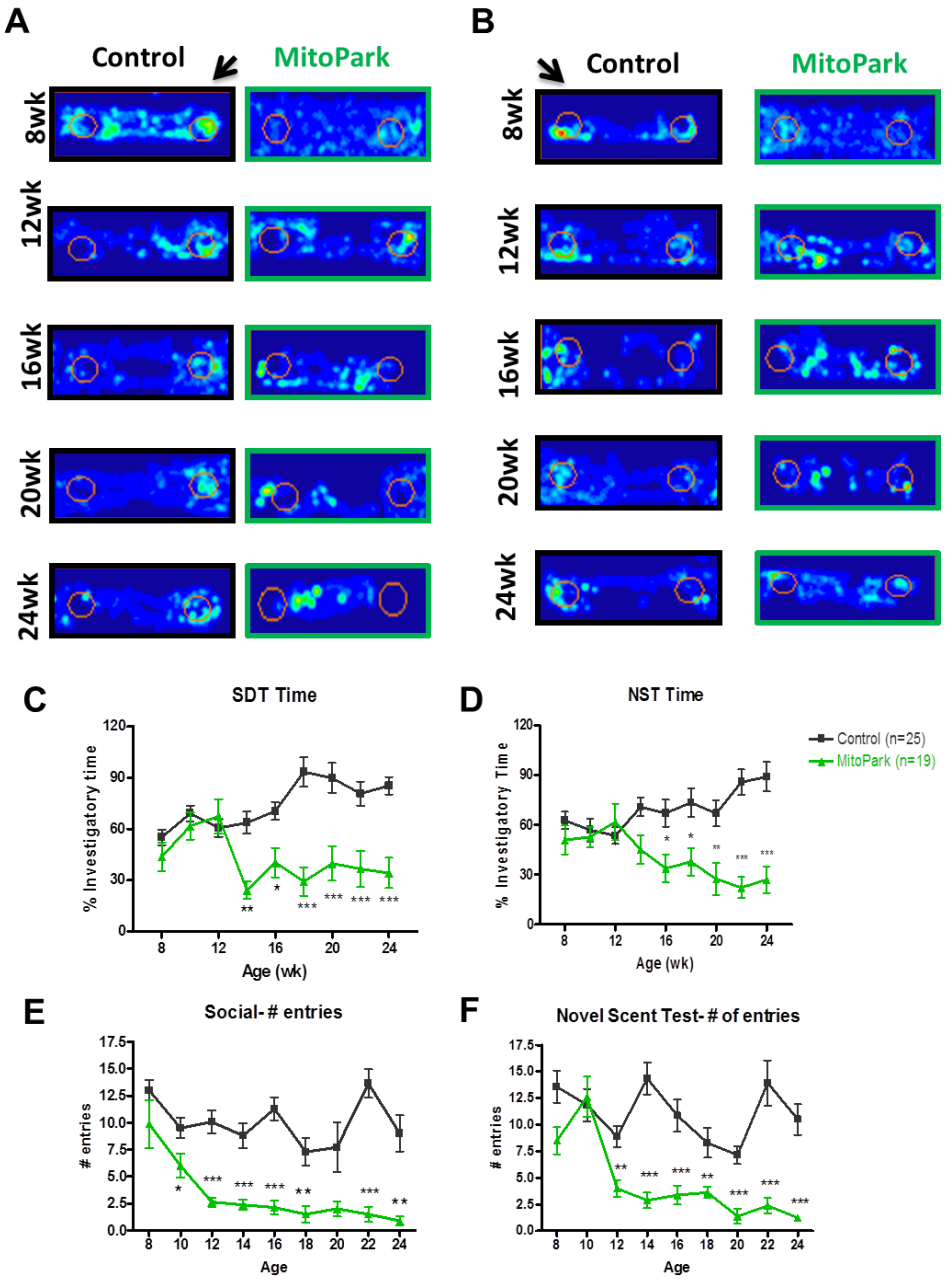

Figure 4. Olfactory dysfunction in MitoPark mice. MitoPark mice were monitored biweekly for olfactory deficits from 8-24 weeks of age. Occupancy plots (*A, B*) and quantification of time in scented zones (*C-F*). Olfactory deficits as determined by social discrimination test (SDT) (*A, C, E*) and novel scent test (NST) (*B, D, F*) were present as soon as 14 weeks of age in MitoPark mice versus controls.

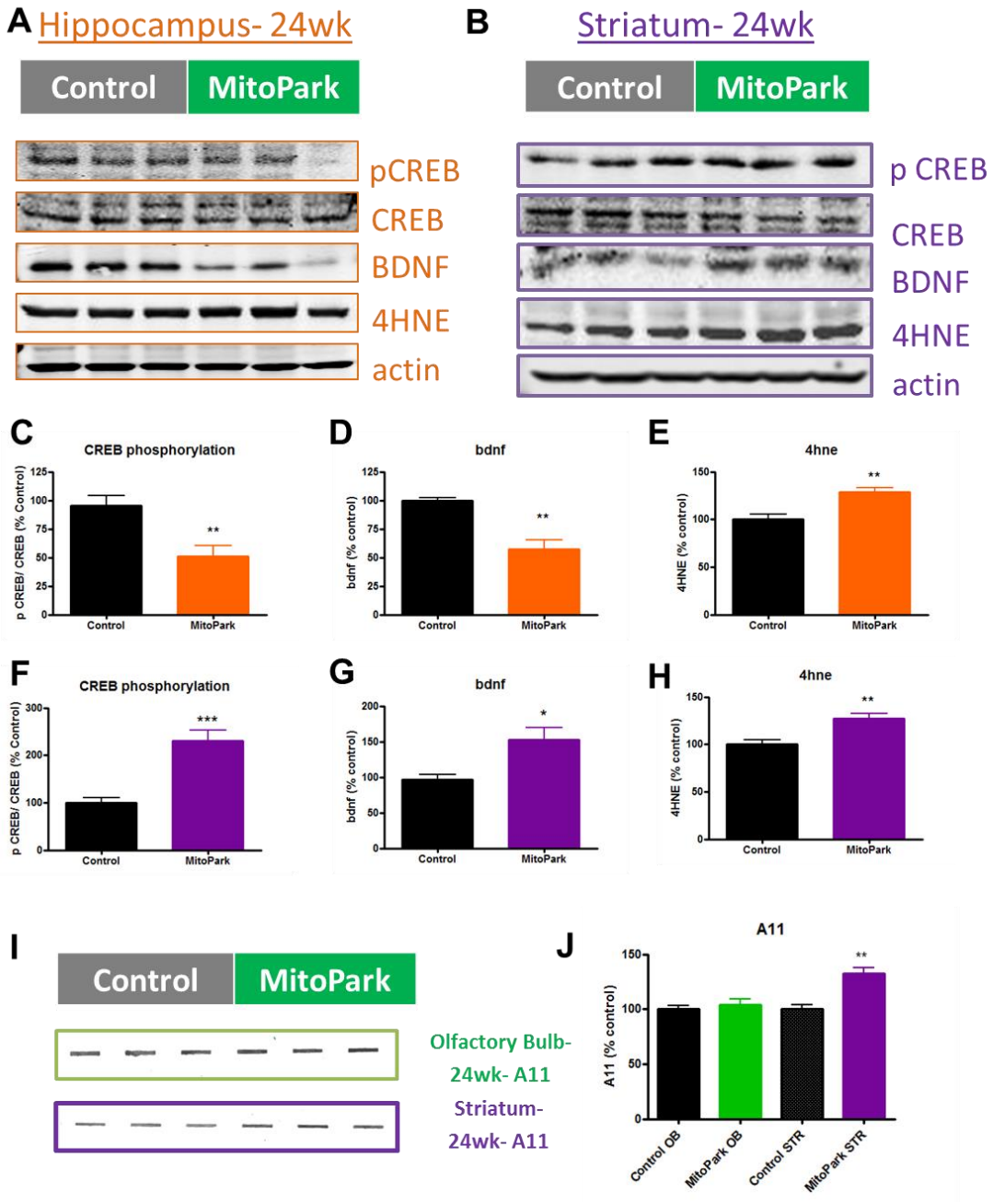

Figure 5. Biochemical changes correlating with observed behavioral symptoms in MitoPark Mice. Western blots (*A*, *B*) and densitometric analysis (*C*-*H*) of proteins related to neuropsychiatric and cognitive changes. A11 slot blot (*I*) and densitometric analysis (*J*) from striatum and olfactory bulb tissues from littermate control and MitoPark mice.

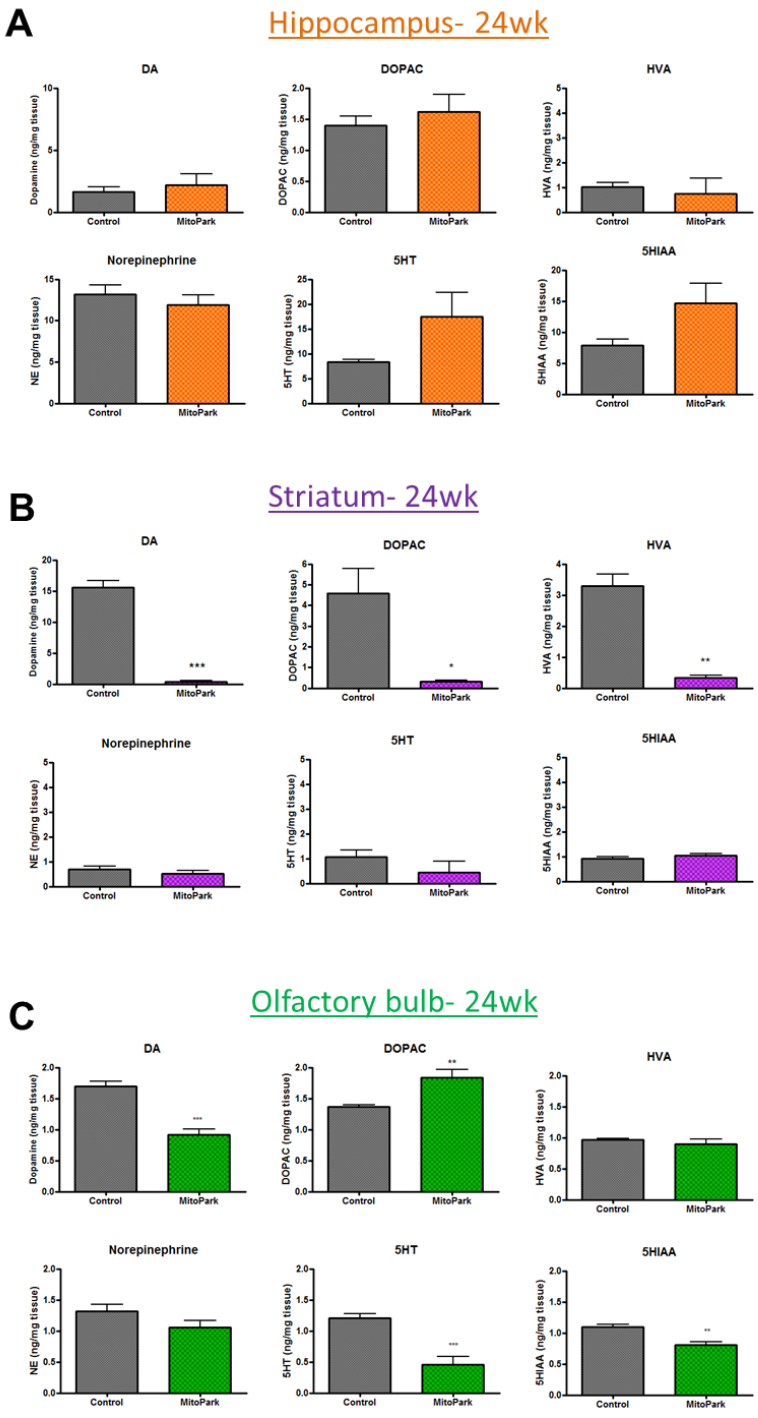

Figure 6. Neurochemical changes in MitoPark mice. Analysis of monoamine neurotransmitters from hippocampus (*A*), striatum (*B*), and olfactory bulb (*C*) tissues from MitoPark mice and their littermate controls at 24-wks of age.

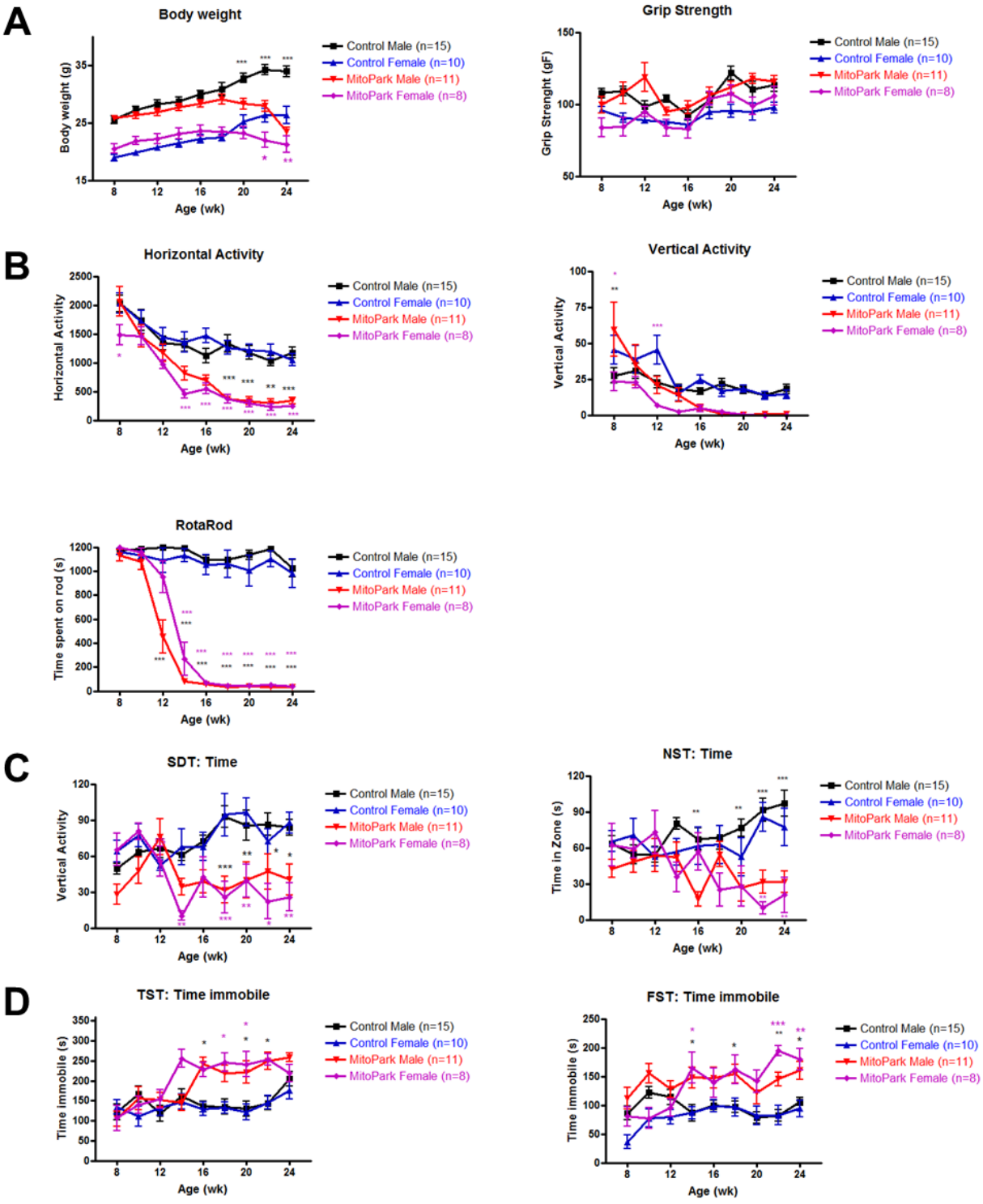

Figure S1. Behavioral data separated by sex.

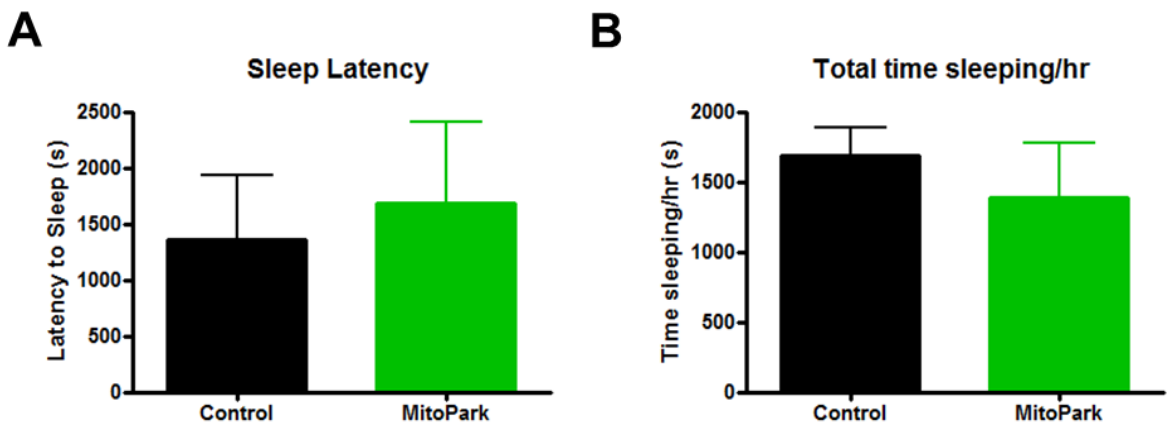

Figure S2. Sleep latency test

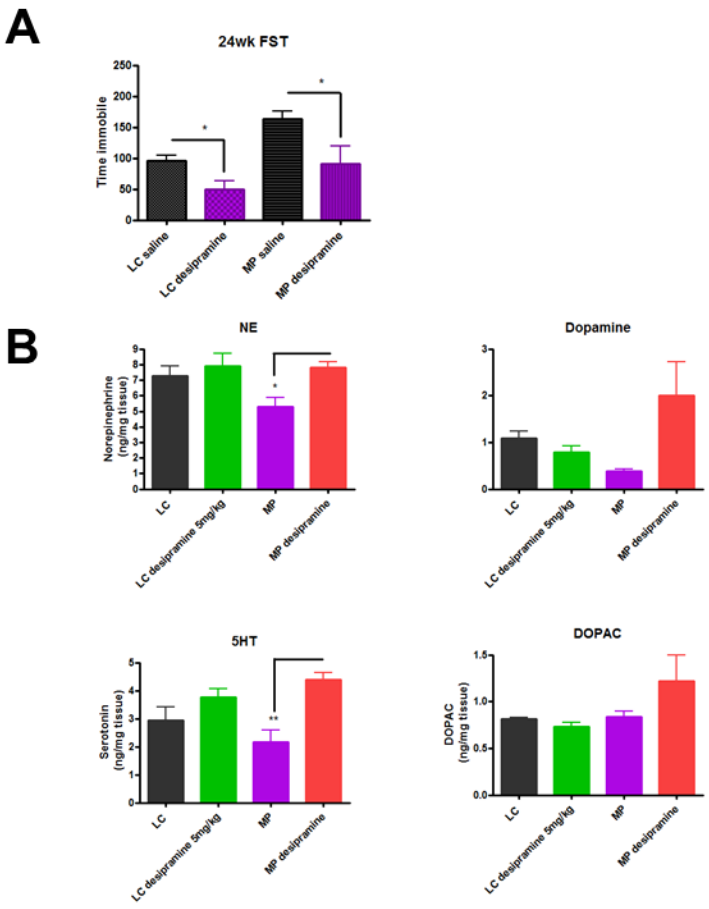

Figure S3. Forced swim test and associated neurochemical data from desipramine treated mice

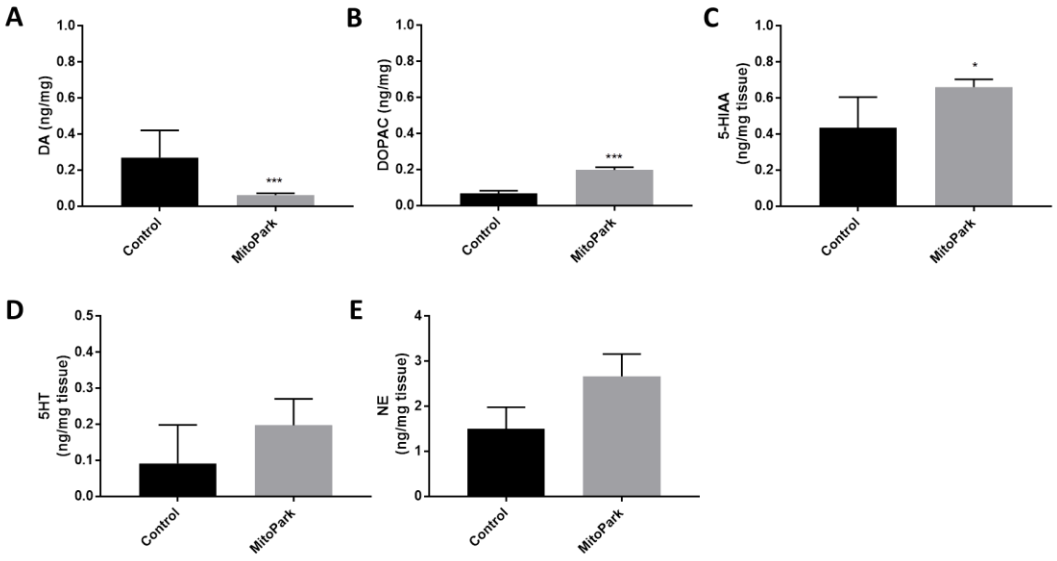

Figure S4. Brainstem neurochemistry

CHAPTER 3

MANGANESE EXPOSURE EXACERBATES PROGRESSIVE MOTOR DEFICITS AND NEURODEGENERATION IN THE MITOPARK MOUSE MODEL OF PARKINSON'S DISEASE: RELEVANCE TO GENE AND ENVIRONMENT INTERACTIONS IN METAL NEUROTOXICITY

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Langley, Monica R.[1]; Ghaisas, Shivani[1]; Ay, Muhammet[1]; Luo, Jie[1]; Palanisamy, Bharathi N.[1]; Jin, Huajun[1]; Anantharam, Vellareddy[1]; Kanthasamy, Arthi[1]; Kanthasamy, Anumantha G.[1]

[1]Parkinson Disorders Research Program, Iowa Center for Advanced Neurotoxicology, Department of Biomedical Sciences, Iowa State University, Ames, IA 50011.

Address correspondence and proof requests to

Dr. A.G. Kanthasamy, Parkinson Disorders Research Laboratory, Department of Biomedical Sciences, 2062 CVM Building, Iowa State University, Ames, IA, 50011

Tel.: (515) 294-2516, Fax: (515) 294 2315, E-mail: akanthas@iastate.edu

Abstract

Parkinson's disease (PD) is now recognized as a neurodegenerative condition caused by a complex interplay of genetic and environmental influences. Chronic manganese (Mn) exposure has been implicated in the development of PD. Since mitochondrial dysfunction is associated with PD pathology as well as Mn neurotoxicity, we investigated whether Mn exposure augments mitochondrial dysfunction and neurodegeneration in the nigrostriatal dopaminergic system using a newly available mitochondrially defective transgenic mouse model of PD, the MitoPark mouse. This unique PD model recapitulates key features of the disease including progressive neurobehavioral changes and neuronal degeneration. We exposed MitoPark mice to a low dose of Mn (10 mg/kg, p.o.) daily for 4 weeks starting at age 8 wks and then determined the behavioral, neurochemical and histological changes. Mn exposure accelerated the rate of progression of motor deficits in MitoPark mice when compared to the untreated MitoPark group. Mn also worsened olfactory function in this model. Most importantly, Mn exposure intensified the depletion of striatal dopamine and nigral TH neuronal loss in MitoPark mice. The neurodegenerative changes were accompanied by enhanced oxidative damage in the striatum and substantia nigra (SN) of MitoPark mice treated with Mn. Furthermore, Mn-treated MitoPark mice had significantly more oligomeric protein and IBA-1-immunoreactive microglia cells, suggesting Mn augments neuroinflammatory processes in the nigrostriatal pathway. To further confirm the direct effect of Mn on impaired mitochondrial function, we also generated a mitochondrially defective dopaminergic cell model by knocking out the TFAM transcription factor by using a CRISPR-Cas9 gene editing method. Seahorse mitochondrial bioenergetic analysis revealed that Mn decreases mitochondrial basal and ATP-linked respiration in the TFAM KO cells.

Collectively, our results reveal that Mn can augment mitochondrial dysfunction to exacerbate nigrostriatal neurodegeneration and PD related behavioral symptoms. Our study also demonstrates that the MitoPark mouse is an excellent model to study the gene-environment interactions associated with mitochondrial defects in the nigral dopaminergic system as well as to evaluate the contribution of potential environmental toxicant interactions in a slowly progressive model of Parkinsonism.

Keywords:

Manganese, mitochondria, MitoPark, neuroinflammation, animal model, TFAM, dopamine, Parkinson's disease, gene-environment interaction

1. Introduction

Although manganese (Mn) is an essential cofactor needed for normal cellular functions, occupational and environmental exposures to the metal have been linked to increased risk for various neurological disorders including Parkinson's disease (PD) (Haynes et al. , 2015, Lucchini et al. , 2014, Sanders et al. , 2015, Sikk and Taba, 2015). Mn exposure commonly occurs during the production of steel, batteries, fuel additives, fireworks, fungicides, welding, and ceramics (Mielke et al. , 2002, Yabuuchi and Komaba, 2014). Given excessive exposure to Mn or failure to excrete it, the metal tends to accumulate in the basal ganglia, resulting in a movement disorder somewhat similar to PD called manganism (Bouabid et al. , 2015, Peres et al. , 2016b). Similar to PD, manganism manifests motor deficits such as rigidity and bradykinesia. However, in manganism patients, a distinctive "cock-walk" gait is observed and neuropsychiatric symptoms often present earlier rather than later in the disease progression (Bowler et al. , 2006, Bowler et al. , 1999, Kwakye et al. , 2015). The neurons most affected in manganism occur in the globus pallidus, rather than the SN (Criswell et al. , 2015, Perl and Olanow, 2007). Furthermore, manganism patients respond poorly to levodopa therapy, so instead chelation therapy has been used to treat the disorder (Discalzi et al. , 2000, Ky et al. , 1992).

Mn and other transition metals have long been implicated as risk factors in the etiology of PD, and a recent study suggests that Mn exposure dose-dependently increases UPDRS3 scores in welders (Gorell et al. , 1999, Racette et al. , 2016). Recent studies showed that welders exposed to Mn fumes display abnormal neurobehavioral changes that correlate with Mn accumulation in the basal ganglia as measured by magnetic resonance imaging (Lee et al. , 2016, Lewis et al. , 2016). Also, chronic exposure to metals and pesticides is

associated with a younger age at onset of sporadic PD (Ratner et al. , 2014). Other studies have revealed that genetic defects in Mn transporter proteins cause metal-induced Parkinsonism, including a form of childhood-onset Parkinsonism caused by an autosomal recessive Mn transporter defect. Patients having the homozygous mutation in SLC39A14 had excessive Mn accumulation and responded positively to chelation therapy (Tuschl et al. , 2016). Furthermore, several mutations in SLC30A10, a Mn-specific efflux transporter thought to protect cells from Mn-induced toxicity, can cause familial Parkinsonism (Chen et al. , 2015a, Leyva-Illades et al. , 2014). The pathogenic mechanisms of Mn neurotoxicity are not completely understood, but evidence suggests that like classical Parkinsonian toxicants, Mn promotes protein aggregation and it also contributes to oxidative stress and mitochondrial dysfunction by inhibiting mitochondrial complexes I and II of the electron transport chain (Aschner et al. , 2009, Carboni and Lingor, 2015, Liu et al. , 2013, Peres et al. , 2016a, Zheng et al. , 1998). Furthermore, Mn can indirectly damage neurons by persistently triggering glial activation and neuroinflammation involving both microglia and astrocytes (Filipov and Dodd, 2012, Moreno et al. , 2011, Moreno et al. , 2009a, Streifel et al. , 2012). Although striatal dopamine (DA) loss is a hallmark of PD models, contradictory results have been found in Mn-treated rodent studies (Moreno et al. , 2009b, Witholt et al. , 2000). These neurochemical changes are thought to contribute to the motor and neuropsychiatric symptoms present (Ferrer et al. , 2012, Vermeiren and De Deyn, 2017).

Growing evidence has indicated that interactions between environmental exposures and genetic factors play a crucial role in the pathogenesis of PD. Mn and many genetic mutations associated with PD alter common biochemical pathways, allowing for a synergistic effect on the development of PD pathology (Bornhorst et al. , 2014, Chen et al. , 2015b,

Roth, 2014). However, little direct evidence exists on Mn exposure exacerbating Parkinsonism in animal models. Such studies could provide much needed insight into how Mn hastens the pathophysiological processes involved in PD, and thus result in the development of better strategies to prevent or delay disease onset and progression. One study that used a toxin-based model to show effects of subchronic intraperitoneal Mn administration on DA-depleted rats reported that Mn potentiated neurobehavioral deficits but not the DA depletion (Witholt, Gwiazda, 2000). Additionally, several PD-related genes such as parkin, LRRK2, and ATP13A2 are known to mediate Mn-induced toxicity in cell culture and animal models (Bornhorst, Chakraborty, 2014, Higashi et al. , 2004, Lovitt et al. , 2010, Tan et al. , 2011). However, none of the neurotoxin-based and genetic PD models faithfully recapitulate the chronic and progressive nature of the disease.

Mitochondrial impairment is well recognized as part of the normal aging process. Moreover, mitochondria serve as a key cellular target for PD pathology as well as for Mn neurotoxicity. However, we lack information on how Mn affects already comprised mitochondrial function as it relates to progression of the neurodegenerative process in PD and aging. Thus, in the present study, we evaluated the effect of a low-dose Mn exposure in the MitoPark mouse, a recently available, mitochondrially defective transgenic (Tg) mouse model of PD. MitoPark mice were generated by conditionally knocking out mitochondrial transcription factor A (TFAM) in DAergic neurons through the Cre-loxP system (Ekstrand et al. , 2007). The MitoPark mouse is a unique PD model that recapitulates most of the hallmark behavioral symptoms and neuropathologies associated with PD, including progressive neurodegeneration and protein aggregates, representing a valuable model for studying the neurodegenerative process. Herein, we utilized the MitoPark mouse for a neurotoxicological

study of Mn using a similar suite of behavioral, neurochemical, histological, and biochemical analyses routinely adopted in other animal models.

2. Materials and Methods

2.1 Chemicals

Dopamine hydrochloride, 3-4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 3,3'-diaminobenzidine (DAB), manganese chloride ($MnCl_2$), and hydrogen peroxide were all purchased from Sigma (St Louis, MO). Halt protease and phosphatase inhibitor cocktail was obtained from Thermo Fisher (Waltham, MA). Bradford assay reagent and Western blotting buffers were purchased from Bio-Rad Laboratories (Hercules, CA). Anti-4-hydoxynonenal (4-HNE) antibody was purchased from R&D Systems (Minneapolis, MN). We purchased anti-IBA-1 antibodies from Wako Pure Chemical Industries (Richmond, VA) and Abcam (Cambridge, MA) for immunohistochemistry (IHC) and Western blot, respectively. Anti-tyrosine hydroxylase (TH) antibody was purchased from Millipore (Billerica, MA). Anti-oligomeric antibody (A11) and cell culture reagents were purchased from Invitrogen. The anti-mouse and anti-rabbit secondary antibodies (Alexa Fluor 680 conjugated anti-mouse IgG and IRdye 800 conjugated anti-rabbit IgG) were purchased from Invitrogen and Rockland Inc., respectively.

2.2 Cell lines

For *in vitro* mitochondrial function studies, the rat immortalized mesencephalic DAergic neuronal cell line ($1RB_3AN_{27}$, or N27) was cultured in RPMI 1640 containing 2 mM $_L$-glutamine, 50 U/ml of penicillin and 50 µg/ml streptomycin with 0-10% FBS in incubators at 37ºC and 5% CO_2 as previously described by our lab (Charli et al. , 2015). The

lentivirus-based CRISPR/Cas9 TFAM knockout plasmid, pLV-U6gRNA-Ef1aPuroCas9GFP-TFAM, with the TFAM gRNA target sequence directed against the exon 1 sequence (CPR555e5e4099bf84.98), was purchased from Sigma-Aldrich. To make lentivirus, the lenti-CRISPR/Cas9 TFAM knockout plasmid was transfected into 293FT cells using the Mission Lentiviral Packaging Mix (Cat#SHP001, Sigma-Aldrich) according to manufacturer's instructions. For negative control lentivirus, Universal Negative Control Lentivirus from Sigma-Aldrich (U6-gRNA/CMV-Cas9-GFP) was similarly transfected into 293FT cells. The lentivirus was harvested 48 h post-transfection and titers were measured using the Lenti-X™ p24 Rapid Titer Kit (Cat # 632200, Takara Bio, Mountain View, CA). For stable knockout of TFAM in N27 cells, cells were plated at 0.1 x 10^6/well and lentivirus was added the following morning to the media at an MOI of 100. After 24 h, fresh media supplemented with puromycin (50 µg/mL) was added to the cells for stable cell selection.

2.3 Animal exposure

MitoPark mice were originally kindly provided by Dr. Nils-Goran Larson, currently at the Karolinska Institute in Stockholm, Sweden, who generated the mouse model in his laboratory at the Max Planck Institute for Biology of Ageing by conditionally knocking out TFAM in cells expressing dopamine transporter (DAT), as described in his publications (Ekstrand, Terzioglu, 2007). All mice for this study were bred, maintained, genotyped, and further characterized at Iowa State University. C57BL/6 mice and MitoPark mice were fed *ad libitum* and housed in standard conditions approved and supervised by the Institutional Animal Care and Use Committee at Iowa State University. Eight-week-old mice (n=7-10/group) received water or 10 mg/kg/day $MnCl_2$ for 30 days by oral gavage. Mice were

weighed and subjected to behavioral tests weekly. Neurochemical, biochemical, and histological studies were performed following sacrifice at 12 wks (Fig. 1A).

2.4 Motor function test

The VersaMax system (VersaMax monitor, model RXYZCM-16, and analyzer, model VMAUSB, AccuScan, Columbus, OH) was used for monitoring locomotor activity and the RotaRod (AccuScan) was used to test coordination of movement as previously described (Ghosh et al. , 2012). For locomotor activity, mice acclimated 2 min prior to monitoring for 10 min using the VersaMax system. RotaRod speed was set to 20 rpm and time spent on rod was measured for 20 min maximum during a total of five trials.

2.5 Social discrimination test

To determine the olfactory function of control and MitoPark mice, we used a social discrimination test as previously described (Ngwa et al. , 2014). However, this procedure was adapted to use AnyMaze tracking software (Stoelting, WoodDale, IL) to determine time spent sniffing based on the animal's head being within a defined zone surrounding the bedding. Total time spent sniffing the opposite sex's bedding (from a group housed cage) was recording during a 3-min interval.

2.6 High performance liquid chromatography (HPLC)

Striatum and olfactory bulb samples were prepared and processed for HPLC as described previously (Gordon et al. , 2016a). Briefly, dissected brain regions were placed in a buffer containing 0.2 M perchloric acid, 0.05% Na2EDTA, 0.1% Na2S2O5, and isoproterenol (internal standard) to extract monoamine neurotransmitters. Monoamine lysates were placed in a refrigerated automatic sampler (model WPS-3000TSL) until being separated isocratically by a reversed-phase C18 column with a flow rate of 0.6 ml/ min using a Dionex

Ultimate 3000 HPLC system (pump ISO-3100SD, Thermo Scientific, Bannockburn, IL). Electrochemical detection was achieved using a CoulArray model 5600A coupled with an analytical cell (microdialysis cell 5014B) and a guard cell (model 5020). Data acquisition and analysis were performed using Chromeleon 7 and ESA CoulArray 3.10 HPLC Software and quantified data were normalized to wet tissue weight.

2.7 Western and slot blots

Protein lysates from the striatum and SN were prepared in RIPA buffer with protease and phosphatase inhibitors. For Western blot, equal amounts of protein lysates were run on a 12-15% SDS-PAGE as previously described (Jin et al. , 2014) and transferred to a nitrocellulose membrane. For slot blot analysis, equal amounts of protein were loaded to each reservoir of the slot blot apparatus (Bio-Dot Microfiltration apparatus, BioRad) and adsorbed to the nitrocellulose membrane as previously described (Harischandra et al. , 2015). After blocking for 1 h, primary antibodies were incubated at 4ºC overnight. The membranes were then incubated with secondary antibodies (Alexa Fluor 680 and Rockland IR800) at RT for one hour and images were captured via Licor Odyssey imager. Densitometric analysis was done using ImageJ software.

2.8 Immunohistochemistry

Brains perfused in 4% paraformaldehyde were cryoprotected with 30% sucrose the following day. Brains embedded in OCT (Sakura Finetek, Torrance, CA) at -80ºC were cryosectioned to 30-µm sections, which were then stored in cryosolution (ethylene glycol and sucrose) until use. Fluoro-Jade C staining was done per manufacturer's instructions (Millipore). Immunostaining was performed as previously described (Gordon, Neal, 2016a). Briefly, following washing, antigen retrieval was achieved by keeping sections at 80ºC for 30

min in sodium citrate (pH 8.5). After blocking in 2% BSA with 0.05% Tween-20 and 0.5% Triton X for one hour, sections were incubated with primary antibodies overnight at 4ºC. For immunofluorescent staining, appropriate secondary antibodies (Alexa Fluor 488 and 555) were selected and incubated with sections for 1.5 h, followed by 7 min incubation with nuclear Hoechst stain (1:5000). For immunoperoxidase staining, a biotinylated secondary antibody followed by incubation with an avidin peroxidase solution (ABC Vectastain Kit, Vector laboratories, Burlingame, CA) to yield a brown stain after incubation with DAB solution. Dehydrated slides were mounted using DPX mountant and images were captured with an inverted Nikon TE2000-U microscope (Tokyo, Japan) attached to a SPOT digital camera with 2-40x lenses (Diagnostic Instruments, Sterling Heights, MI).

2.9 Statistics

All statistical analysis was done by repeated measures two-way ANOVA with Bonferroni post-test, one-way ANOVA with Dunnett's post-test, Student's t-test, or linear regression using GraphPad Prism. Differences with p-values <0.05 were considered significant.

3. Results

3.1 Low-dose Mn exposure accelerates the progressive behavioral deficits in MitoPark mice

MitoPark Tg mice display an adult-onset motor impairment, beginning around age 14 wks. Symptoms, including tremor and rigidity, progressively worsen by 20 wks (Ekstrand, Terzioglu, 2007). Therefore, we began our study with mice at age 8 wks, prior to motor deficits in this model. Similarly, we chose a dose of Mn (10 mg/kg) that has been shown not to produce significant changes in behavioral parameters or striatal neurotransmitter levels in

healthy adult mice (Moreno, Yeomans, 2009b). Therefore, we administered water or 10 mg/kg Mn by oral gavage daily to MitoPark and littermate control mice from ages 8-12 wks (Figure 1A). Behavioral tests were performed weekly, and biochemical and neurochemical analyses were performed on mice sacrificed at age 12 wks.

We first evaluated the locomotor activity and coordination of C57 and MitoPark mice using VersaMax and RotaRod tests, respectively. Representative activity plots obtained from open-field test at age 12 wks revealed a reduction in activity in Mn, MitoPark, and MitoPark-Mn groups when compared to C57 age-matched controls (Figure 1B). Linear regression of horizontal activity against age shows that Mn-treated C57 mice became significantly less active over time ($p=0.0068$) (Figure 1C). However, MitoPark mice treated with Mn had a more significant reduction in horizontal activity over time ($p<0.001$). Similarly, Mn-treated MitoPark mice experienced a more significant decline in vertical activity (Figure 1D, $p=0.0425$, Mn; $p=0.0183$, MitoPark; $p<0.0001$, Mn-treated MitoPark). Moreover, Mn-treated MitoPark mice were the only group that spent less time on the 20-rpm RotaRod (Figure 1E). Although both MitoPark controls and Mn-treated MitoParks traveled less distance during the 10-min interval, the Mn-treated MitoParks showed an earlier and more severe deficit (Figure 1F, $p=0.0035$, MitoPark-Control; $p=0.0002$, MitoPark-Mn). C57 and MitoPark mice treated with Mn had reductions in rearing activity over time (Figure 1G, $p=0.0438$, C57-Mn; $p<0.0001$, MitoPark- Mn).

Previous studies in our lab and others have indicated that olfactory dysfunction, which is a nonmotor symptom of PD that can precede onset of motor symptoms by many years, is inherent in experimental models of PD and Mn neurotoxicity (Dranka et al. , 2014, Neuner et al. , 2014, Ngwa, Kanthasamy, 2014, Zhang et al. , 2015). We therefore performed

a social discrimination test to examine the effects of Mn exposure on olfaction. MitoPark mice did not show significantly decreased olfaction at age 12 wks, however, both Mn-treated C57 and Mn-treated MitoParks spent significantly less time sniffing the opposite-sex bedding during a 3-min social discrimination test (Figure 1H).

Overall, many locomotor deficits present in MitoPark mice were exacerbated and accelerated by Mn exposure. Coordination and rearing activity were not yet impaired in MitoPark mice by age 12 wks, but were significantly affected in MitoPark mice exposed to Mn (Table 1). In contrast, Mn exposure impaired olfaction in both C57 and MitoPark mice. These results suggest that, unlike the effect of Mn on motor deficits, Mn-induced olfactory deficits do not differ between C57 and MitoPark mice. Thus, mitochondrial impairment may not significantly contribute to the development of olfactory deficits.

3.2 Mn exacerbates striatal DA depletion and DAergic neuronal loss in MitoPark Tg mice

After establishing the behavioral effects of Mn in MitoPark mice, we next determined the effects on the nigrostriatal tract. In MitoPark mice, striatal DA loss is first observed at age 12 wks with only about 60% of TH^+ neurons remaining at that time (Ekstrand, Terzioglu, 2007). We first examined whether Mn exposure affects neurochemical deficits in the striatum by measuring DA, DOPAC and HVA by HPLC with electrochemical detection. Although MitoPark mice showed significantly less DA and metabolites than C57 controls, Mn exposure worsened the depletion of DA (Figure 2A), DOPAC (Figure 2B), and HVA (Figure 2C) in the striatum of MitoPark mice. To further determine the effects of Mn on DAergic neuronal degeneration in the nigrostriatal tract, we performed TH IHC analyses on 30-μm sections of the striatal and nigral regions from 12-wk C57 and MitoPark mice treated with

water or Mn. No significant reduction in TH-immunoreactivity was observed in vehicle- and Mn-treated non-Tg controls (Figure 2D). However, MitoPark mice did show less TH-immunoreactivity in nigral cell bodies and striatal terminals (Figure 2D). Importantly, Mn exposure exacerbated the loss of TH^+ cells in the SN and immunostaining in the striatum in MitoPark mice (Figure 2D). Taken together, our neurochemical and histological data clearly demonstrate that Mn exposure exacerbates the neurodegenerative process in DAergic neurons.

3.3 Mn increases oxidative stress in the brains of MitoPark mice

Since Mn is known to induce oxidative damage, we assessed Mn-induced oxidative damage in MitoPark mice by measuring levels of 4-HNE, a lipid peroxidation product (Abdul-Muneer et al. , 2013, Ghosh et al. , 2016, Seo et al. , 2016). As evidenced by Western blotting, 4-HNE was significantly increased in the SN (Figure 3A) and striatum (Figure 3B) of Mn-treated MitoPark mice. Consistently, double-IHC shows increased 4-HNE immunoreactivity (Figure 3C) in TH^+ neurons in the SN of Mn-treated MitoPark mice, while no significant changes in 4-HNE were detectable in Mn-treated C57 or MitoPark-Control mice. These data suggest that low-dose Mn exposure and the inherent mitochondrial dysfunction in the MitoPark model combine to exacerbate oxidative stress in the nigrostriatal tract.

3.4 Mitochondrial dysfunction and neuronal cell death in Mn-treated MitoPark mice

MitoPark mice show decreased expression of the mtDNA encoded COX subunit I (MTCO1), indicating a severe reduction in mtDNA expression that results in respiratory chain deficiency in DAergic neurons (Ekstrand, Terzioglu, 2007). We observed significantly reduced protein levels of MTCO1 in the striatum (Figure 4A) of MitoPark mice as well as

Mn-treated MitoPark mice. A similar reduction trend in MTCO1 protein levels, although not statistically significant, was observed in the SN (Figure 4B). As inherent mitochondrial dysfunction eventually results in the death of DAergic neurons in the MitoPark model, we next examined neuronal cell death by Fluoro-jade staining (Figure 4C), which revealed more degenerating neurons in the SN of MitoPark mice when compared to C57 Controls. However, a robust increase in Fluoro-Jade-labeled cells in the Mn-treated MitoPark mice suggests an enhanced cell death occurring in the Mn-treated MitoPark mice.

3.5 Mn induces neuroinflammatory response MitoPark mice

Neuroinflammation has been identified as an important contributor to neurodegenerative processes in PD. Although glial activation has not yet been characterized in the MitoPark model, it is understood that damaged neurons secrete factors which can activate glial cells (Block and Calderon-Garciduenas, 2009, Dhawan and Combs, 2012, Gordon et al. , 2016b, Levesque et al. , 2010, Panicker et al. , 2015). Furthermore, Mn has been shown to promote microglia activation in various model systems (Kraft and Harry, 2011, Moreno, Streifel, 2009a, Park and Chun, 2016). Western blotting revealed significantly more IBA-1 protein in the SN of Mn-treated MitoPark mice. However, neither Mn-treated C57or MitoPark-Controls had a significant elevation in IBA-1 (Figure 5A). These results are consistent with DAB-immunostaining showing more IBA-1^{+} cells in the SN of Mn-treated MitoParks (Figure 5B). Together, these results imply that Mn exposure in MitoPark mice can induce neuroinflammation in the SN, which may further contribute to neurodegeneration.

3.6 Mn increases protein aggregation in MitoPark mice

Even prior to the symptomatic stage, MitoPark mice were found to contain protein aggregates in their DAergic neurons (Ekstrand, Terzioglu, 2007). Although these aggregates

did not contain α-synuclein, they did increase in size over time and partially co-localized with mitochondrial membrane proteins (Ekstrand, Terzioglu, 2007). Our lab has shown that Mn enhances oligomerization of proteins in PD and prion models (Choi et al. , 2006, Harischandra, Jin, 2015, Rokad et al. , 2016). We therefore examined the levels of protein aggregation by slot blot analysis with an oligomeric-specific antibody, A11. Although A11 modestly increased in C57-Mn and MitoPark groups, only the Mn-treated MitoPark group showed significantly increased oligomeric protein levels when compared to C57 control mice (Figure 6A). Overall, these results provide evidence that Mn enhances protein aggregation in MitoPark mice.

3.7 Mn potentiates mitochondrial deficits in a TFAM-KO DAergic neuronal cell model

Mitochondrial biogenesis is controlled by the transcription factor TFAM. Similar to MitoPark mice, knocking out TFAM produces defective mitochondria. To further confirm that Mn directly impairs mitochondrial dynamics in DAergic neuronal cells with defective mitochondria, we created a stable TFAM knockout (KO) N27 cell line using a CRISPR/Cas9-based lentiviral system. Our qRT-PCR analysis revealed more than an 80% loss of TFAM mRNA levels in TFAM-KO cells compared to CRISPR control cells (data not shown). Mitochondrial dynamics were then assessed by using a Seahorse extracellular flux analyzer. We exposed both TFAM-KO and control N27 neuronal cells to a low concentration of Mn (100 μM) for 24 h. This concentration of Mn alone did not result in mitochondrial deficits since both basal respiration rate and ATP-linked respiration did not significantly differ between vehicle- and Mn-treated control cells (Figure 7A-C). As expected, TFAM KO reduced basal respiration rate and ATP-linked respiration in untreated N27 cells. Interestingly, further reductions in basal respiration were observed in Mn-treated TFAM-KO

N27 cells (Figure 7A, B). Following oligomycin exposure, lower ATP-linked respiration was also observed in the Mn-treated TFAM-KO group (Figure 7C). Taken together, these data suggest that Mn augments mitochondrial dysfunction by impairing both basal and ATP-linked respiration capacity in a DAergic neuronal cell model.

4. Discussion

In the present study, we systematically characterized neurobehavioral, neurochemical and biochemical changes contributing to nigral DAergic neurodegeneration in a transgenic, mitochondrially defective animal model (MitoPark mice) exposed to Mn. We demonstrated that orally administering low-dose Mn increased microglia activation and the formation of 4-HNE in MitoPark mice, suggesting that combining both genetic deficits and exposure to an environmental neurotoxic metal synergistically accentuates oxidative and inflammatory processes. Importantly, Mn exposure also accelerated and exacerbated motor deficits, the formation of aggregated proteins, and nigrostriatal DAergic degeneration in MitoPark mice. This is the first report, to our knowledge, describing a synergistic interaction between environmental Mn exposure and inherent mitochondrial dysfunction in accelerating the development of PD pathology.

Although neuropsychiatric symptoms of Mn at high doses are well researched, not much is known about low-dose Mn exposure and its neurobehavioral outcomes. A study by Moreno et al. (2009b) has shown that low-dose Mn alters anxiolytic behavior in male mice. Anxiolytic and depressive phenotyping were outside the scope of this paper, but would provide valuable insight and should be explored in future studies. In terms of non-motor deficits, olfactory deficits found in Mn-treated mice during this study (Figure 1H) could potentially be due to increased protein aggregation, reductions in neurogenesis, or changes in

the olfactory epithelium or receptors (Chiu et al. , 2015, Kurtenbach et al. , 2013, Neuner, Filser, 2014, Postuma and Berg, 2016).

The pathophysiological mechanisms underlying Mn-induced exacerbation of DAergic neurons are not exactly clear. However, the increase in degenerating neurons and subsequent symptoms are likely a result of a combination of pathological pathways being further perturbed by Mn exposure. We have provided evidence that oxidative stress, protein aggregation, and neuroinflammation play a role in the Mn-mediated acceleration of disease progression in the MitoPark model. Furthermore, the effects that the interaction of these pathways could have on other processes should not be ignored. For example, the increased oxidative stress and protein aggregation observed could contribute to the gliosis found in Mn-treated MitoPark mice (Mosley et al. , 2006, Zhang et al. , 2005). Given the progressive phenotype, older-aged MitoPark mice should be characterized to explore the possibility of reactive microgliosis in this model in the absence of Mn exposure. It would be interesting to decipher whether mitochondrial dysfunction in the MitoPark model was exacerbated by the Mn exposure or rather by an increased sensitivity to Mn toxicity in this model. Our results from TFAM-KO cells (Figure 7) suggest that Mn exposure directly impairs basal mitochondrial oxygen consumption rate and ATP-linked respiration. Also, the possibility of transport or excretion of Mn being compromised in this model cannot be ruled out. Further characterization of the biochemical mechanisms in MitoPark mice may help to address such questions.

Electrophysiological parameters in DAergic neurons were found to be affected in MitoPark mice, even prior to motor deficits (Branch et al. , 2016). Because Mn enhances oxidative stress by catalyzing the auto-oxidation of DA and participating in Haber-Weiss

reactions, this could explain the potent increase in lipid peroxidation (Figure 3) (Carboni and Lingor, 2015, Farina et al. , 2013). Moreover, Mn is thought to preferentially accumulate in DAergic neurons due to increased calcium channel expression allowing for increased uptake of Mn (Carboni and Lingor, 2015). Branch and colleges (2016) revealed increased mRNA levels of Cav1.2 subunits in the 12-week-old MitoPark mouse, and more upregulation of ion channel subunit mRNAs associated with spontaneous firing at age 18-22 wks. In the mitochondria, Mn can inhibit mitochondrial aconitase and complexes I and II of the electron transport chain (Liu, Barber, 2013, Zheng, Ren, 1998). We previously reported that Mn exposure activates a mitochondria-dependent apoptotic cell death pathway in the DAergic system by a PKCδ-dependent proteolytic activation (Anantharam et al. , 2004, Anantharam et al. , 2002, Kitazawa et al. , 2005, Kitazawa et al. , 2002, Latchoumycandane et al. , 2005). Thus, multiple mechanisms may be involved in regulating the Mn-induced sensitization of mitochondrial impairment. Further studies in in vitro and in vivo models of mitochondrial defects will provide additional mechanistic insights into Mn-induced neurodegeneration. Gene-environment interactions are difficult to determine largely due to challenges in estimation of environmental exposures to toxicants and potential confounding factors (Polito et al. , 2016, Thomas, 2010). A better understanding, however, could provide for novel biomarkers, therapeutic strategies, and personalized medicine. In the MitoPark mouse model, mitochondrial dysfunction is targeted to cells expressing DAT (Ekstrand, Terzioglu, 2007). A number of studies have suggested that combining genetic variability in the DAT gene (SLC6A3) with pesticide exposure could interact synergistically to increase odds-ratios in PD patients (Kelada et al. , 2006, Polito, Greco, 2016, Ritz et al. , 2009, Ritz et al. , 2016, Singh et al. , 2008). Investigations of Mn and welding in relation to PD have led to

controversial and opposing outcomes in various studies. Some studies report an increased PD risk, while others either do not report additional risk or emphasize the difference between PD and Mn-induced Parkinsonism (Guilarte and Gonzales, 2015, Nandipati and Litvan, 2016, Polito, Greco, 2016, Rentschler et al. , 2012, Sriram et al. , 2010). Multiple PARK genes have been shown to increase Mn susceptibility in model systems and epidemiological data (Bornhorst, Chakraborty, 2014, Carboni and Lingor, 2015, Higashi, Asanuma, 2004, Lovitt, Vanderporten, 2010, Rentschler, Covolo, 2012, Tan, Zhang, 2011). For instance, a study using induced pluripotent stem cell-derived neural progenitor cells showed significantly higher ROS generation in cells from a human subject with mutated PARK2 than in control subject-derived cells following Mn exposure, indicating increased sensitivity (Aboud et al. , 2012). Other PARK genes are thought to function as Mn transporters (Carboni and Lingor, 2015, Tuschl, Meyer, 2016). Exceptionally little data can be found linking epigenetic changes induced by Mn to effects on PD pathological mechanisms (Peng et al. , 2015). Mn has also been implicated in gene-environment interactions in Huntington's and Alzheimer's diseases (Aboud, Tidball, 2012, Bornhorst, Chakraborty, 2014, Chin-Chan et al. , 2015, Madison et al. , 2012, Tong et al. , 2014). Thus, continued efforts in studying the effect of environmental neurotoxicants in genetic defect models will shed light on gene-environment interactions in environmentally linked neurodegenerative diseases.

In conclusion, we show that low-dose Mn exposure significantly accelerates and exacerbates the motor deficits, striatal dopamine depletion and TH neuronal loss in MitoPark mice, demonstrating that Mn exposure can augment neurodegenerative processes in sensitive populations with mitochondrial deficiency, including the elderly community. Furthermore, our data demonstrate the utility of the MitoPark model for gene-environment interaction

studies as well as for studying neurotoxic mechanisms in potentiating Parkinsonism in transgenic mouse models. The pathological role of Mn in other genetic models of PD and novel mitochondria-targeted therapeutic strategies for Mn-induced Parkinsonism should be further explored.

Conflict of interest:

A.G.K. and V.A. are shareholders of PK Biosciences Corporation (Ames, IA), which is interested in developing therapeutic strategies for neurodegenerative diseases including PD and manganism. Other authors declare no potential conflicts of interest.

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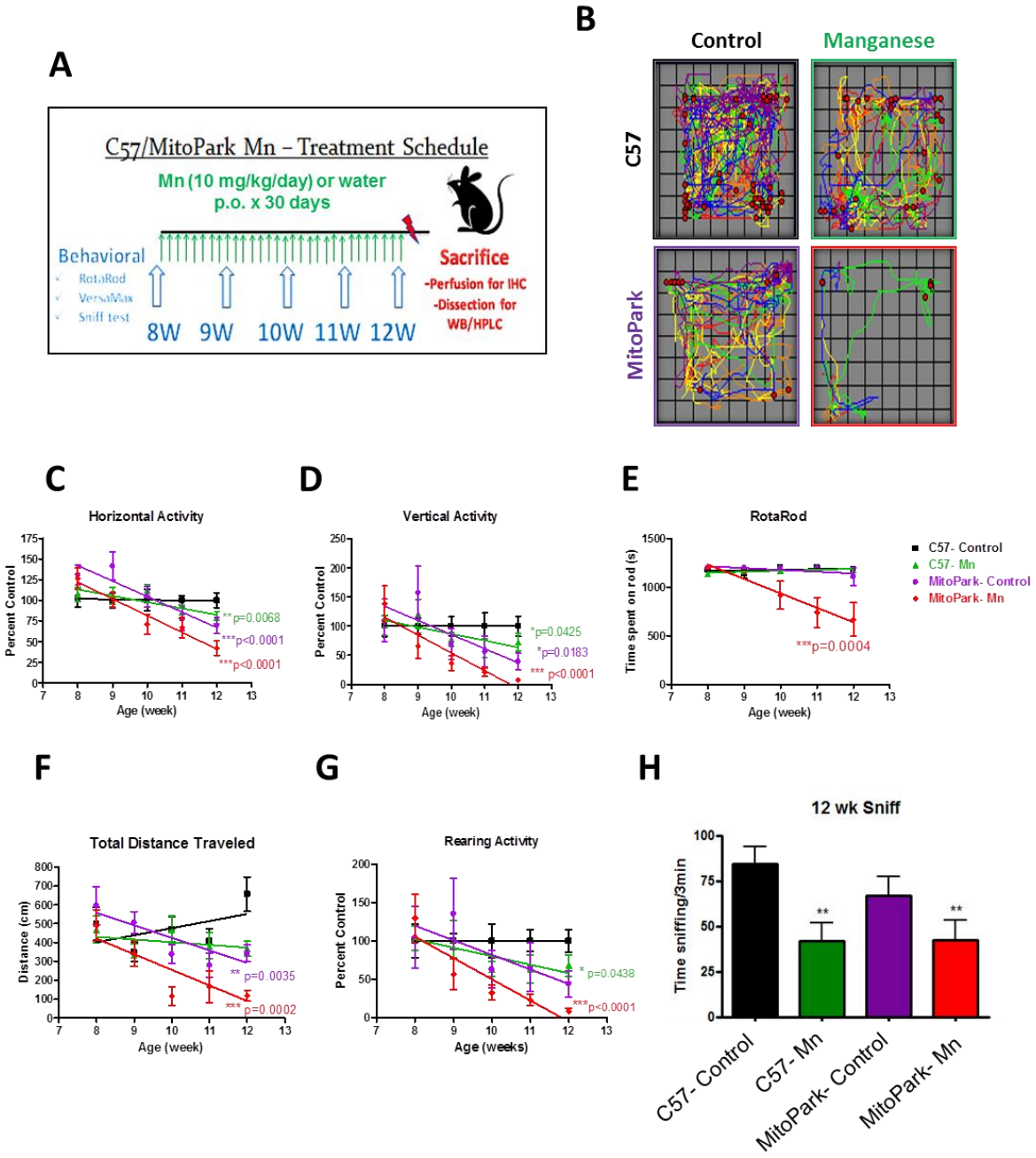

Figure 1. Mn accelerates and exacerbates progressive behavioral deficits in MitoPark mice. *A*, Exposure schedule showing C57 and MitoPark mice orally administered water or Mn (10 mg/kg) from ages 8-12 wks. *B*, VersaPlot showing horizontal activity (lines) and rearing activity (red dots) during a 10-min open-field test. Horizontal (*C*), vertical (*D*), and

rearing (*G*) activities and distance traveled (*F*) during open-field test. *E*, Time spent on RotaRod. *H*, Time spent sniffing scented zone during 3-min social discrimination test. Graphical results represented as the mean±SEM (n=7-10 mice/group). *, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$ versus water-treated C57 Control.

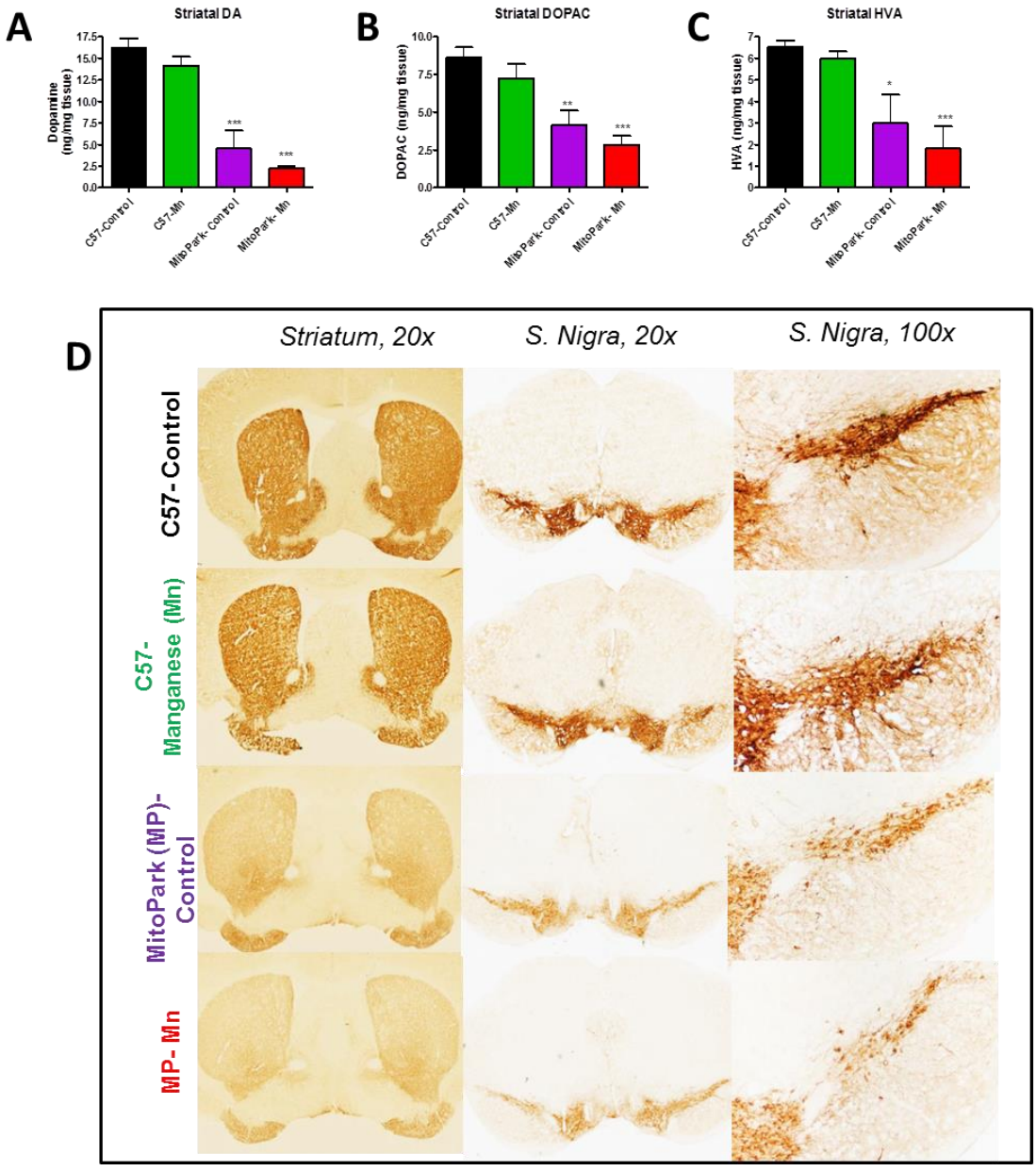

Figure 2. Mn exacerbates striatal DA depletion and TH neuronal loss MitoPark mice

HPLC with electrochemical detection of the neurotransmitters dopamine (A), DOPAC (B), and HVA (C) in the striatum. DAB immunostaining (D) was performed in striatum and substantia nigra of vehicle-treated C57 (top), Mn-treated C57 (second row), vehicle-treated

MitoPark (MP) (third row) and Mn-treated MP mice (bottom). Graphical results represented as the mean±SEM (n=7-10 mice/group). *, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$ versus water-treated C57 Control.

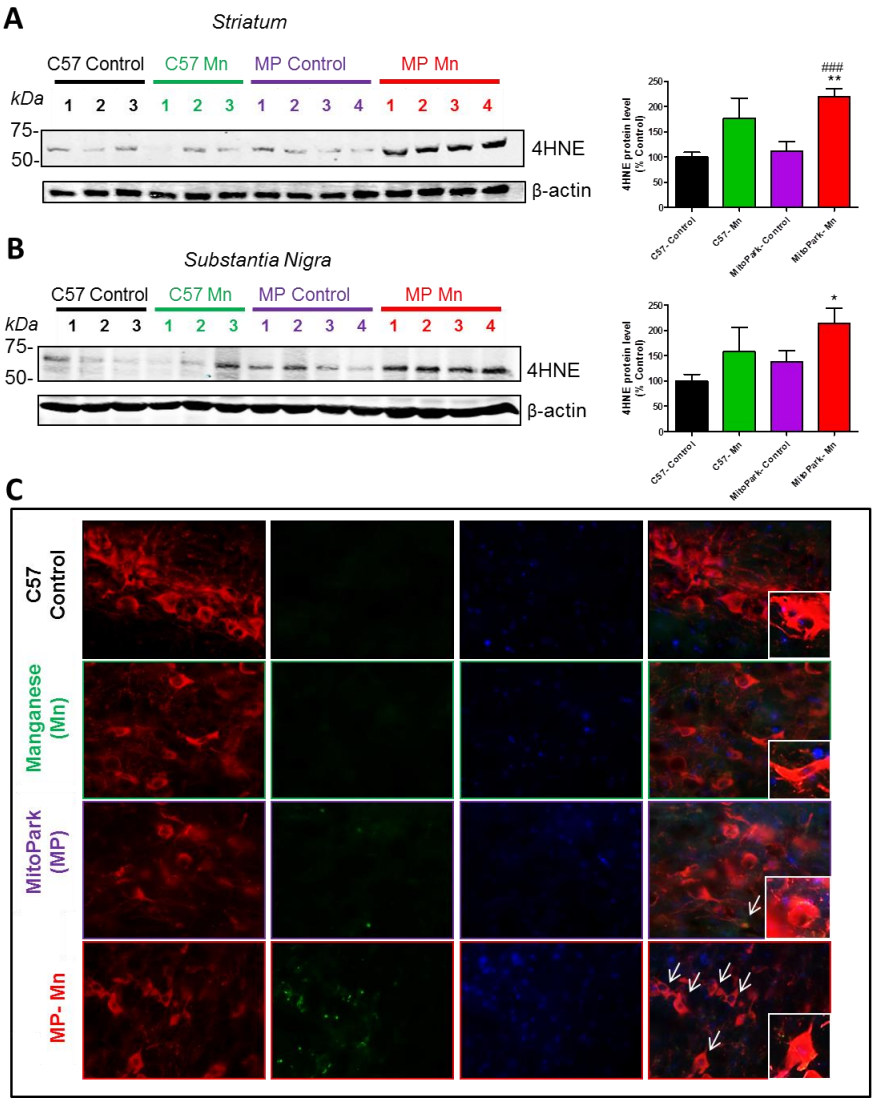

Figure 3. Mn increases oxidative stress in the brains of MitoPark mice. Representative Western blots and densitometric analysis of 4-HNE protein in the striatum (*A*) and substantia nigra (*B*). Immunohistochemistry of 12-wk mouse substantia nigra (*C*) reveals co-localization of TH and 4-HNE in dopaminergic neurons of Mn-treated MitoPark mice. Graphical results represented as the mean±SEM (n=7-10 mice/group). *, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$ versus water-treated C57 Control. ###, $P<0.001$ versus water-treated MitoPark.

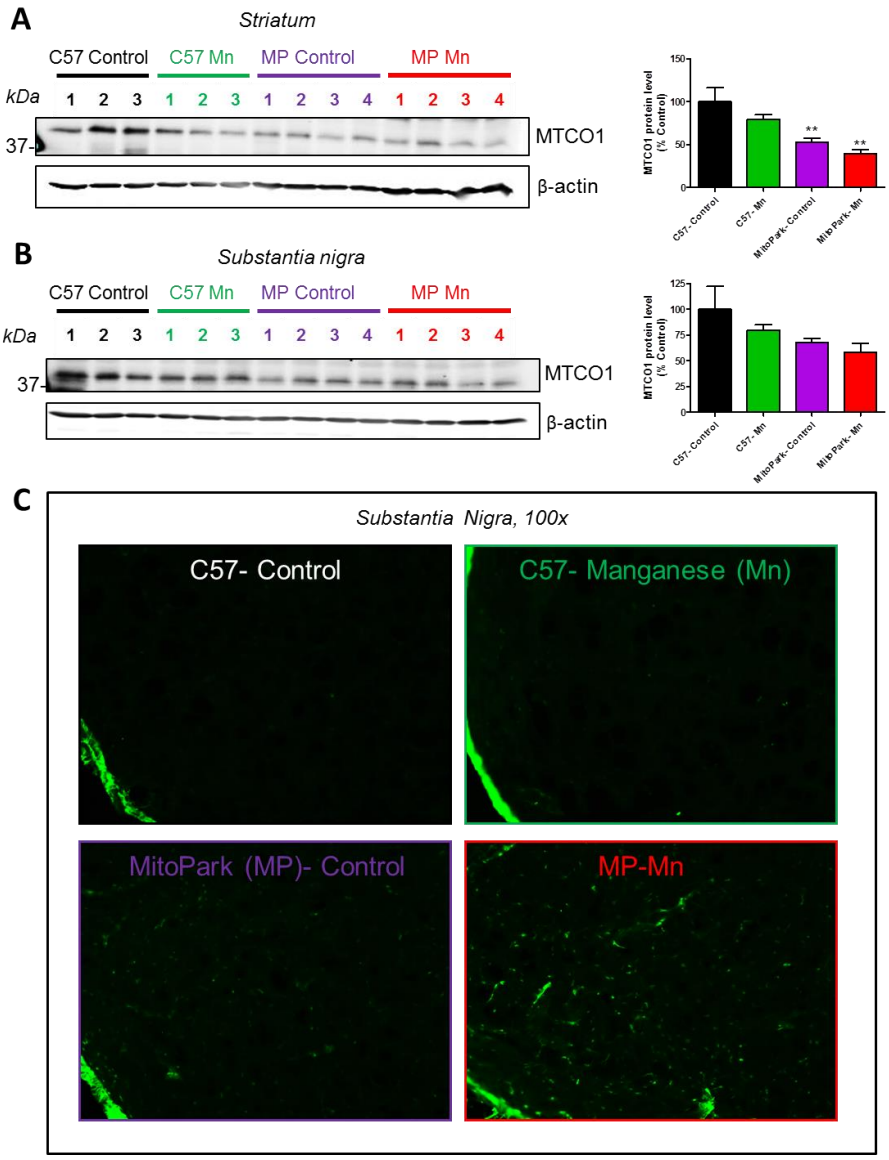

Figure 4. Mitochondrial dysfunction and neuronal cell death in Mn-treated MitoPark mice. Representative Western blots and densitometric analysis of MTCO1 protein in the striatum (*A*) and substantia nigra (*B*). Fluoro-Jade C staining of 12-wk mouse substantia nigra (*C*) reveals increased neuronal cell death in Mn-treated MitoPark mice. Graphical results represented as the mean±SEM (n=7-10 mice/group). *, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$ versus water-treated C57 Control.

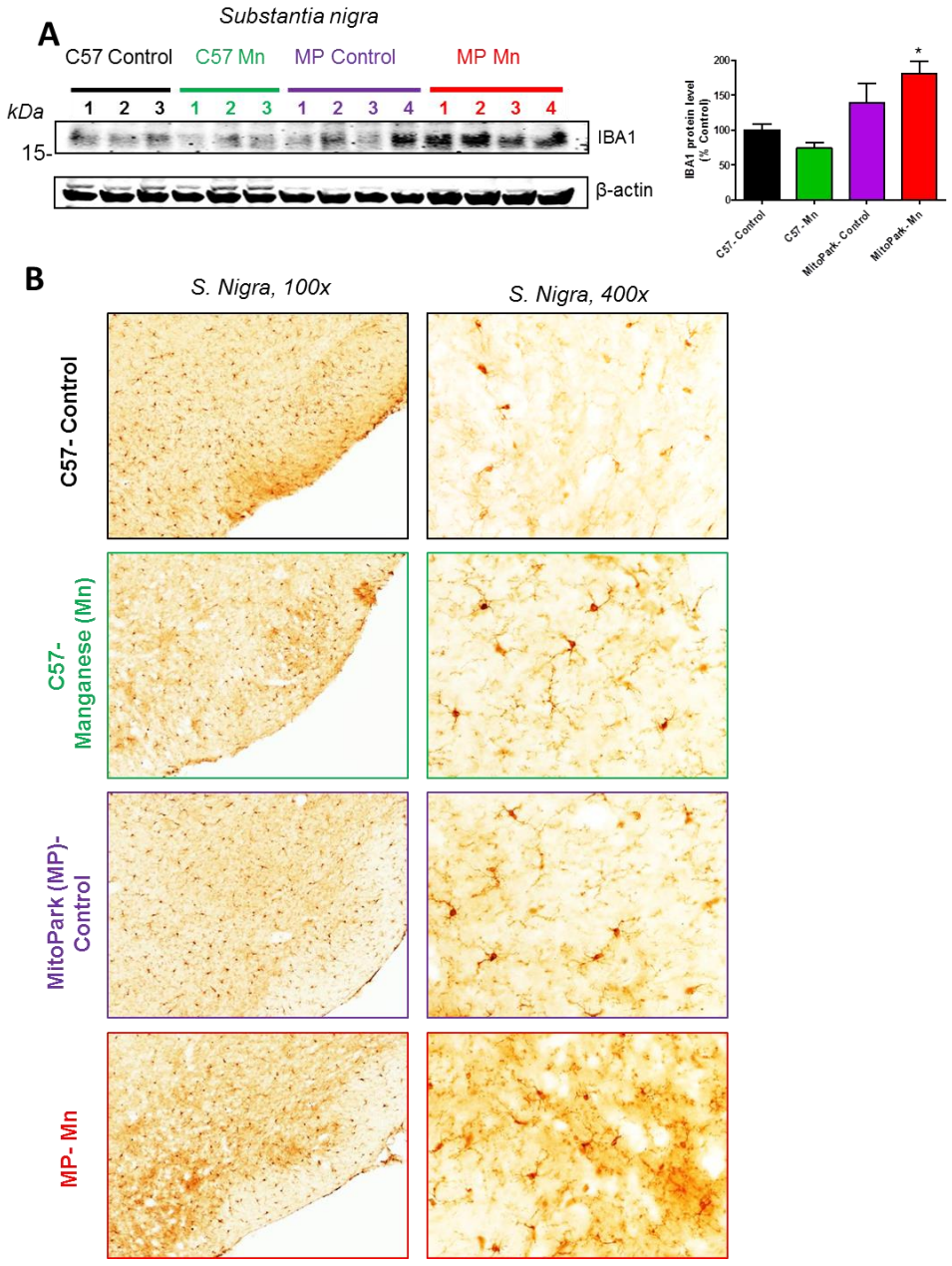

Figure 5. Neuroinflammatory changes in Mn-treated MitoPark mice

A, Western blot and corresponding densitometric analysis of IBA-1 protein in the SN. B, IBA-1 DAB immunostained sections from 12-wk mouse substantia nigra of vehicle-treated C57 (top), Mn-treated C57 (second row), vehicle-treated MitoPark (MP) (third row) and Mn-treated MP mice (bottom) show more IBA-1^{+} microglia in Mn-treated MitoPark mice when

compared to vehicle-treated C57 Control mice. Graphical results represented as the mean±SEM (n=7-10 mice/group). *, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$ versus water-treated C57 Control.

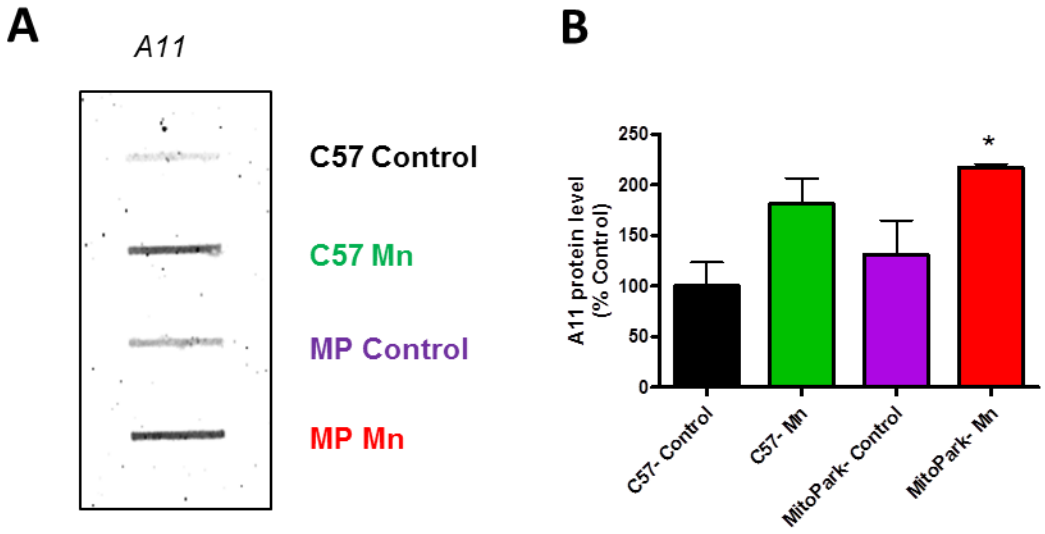

Figure 6. Mn increases protein aggregation in MitoPark mice

A, Representative slot blot analysis for oligomeric protein-specific antibody (A11) shows more oligomeric protein present in the substantia nigra of Mn-treated MitoPark mice. *B,* Quantification of A11 slot blot. Graphical results represented as the mean±SEM (n=7-10 mice/group). *, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$ versus water-treated C57 Control.

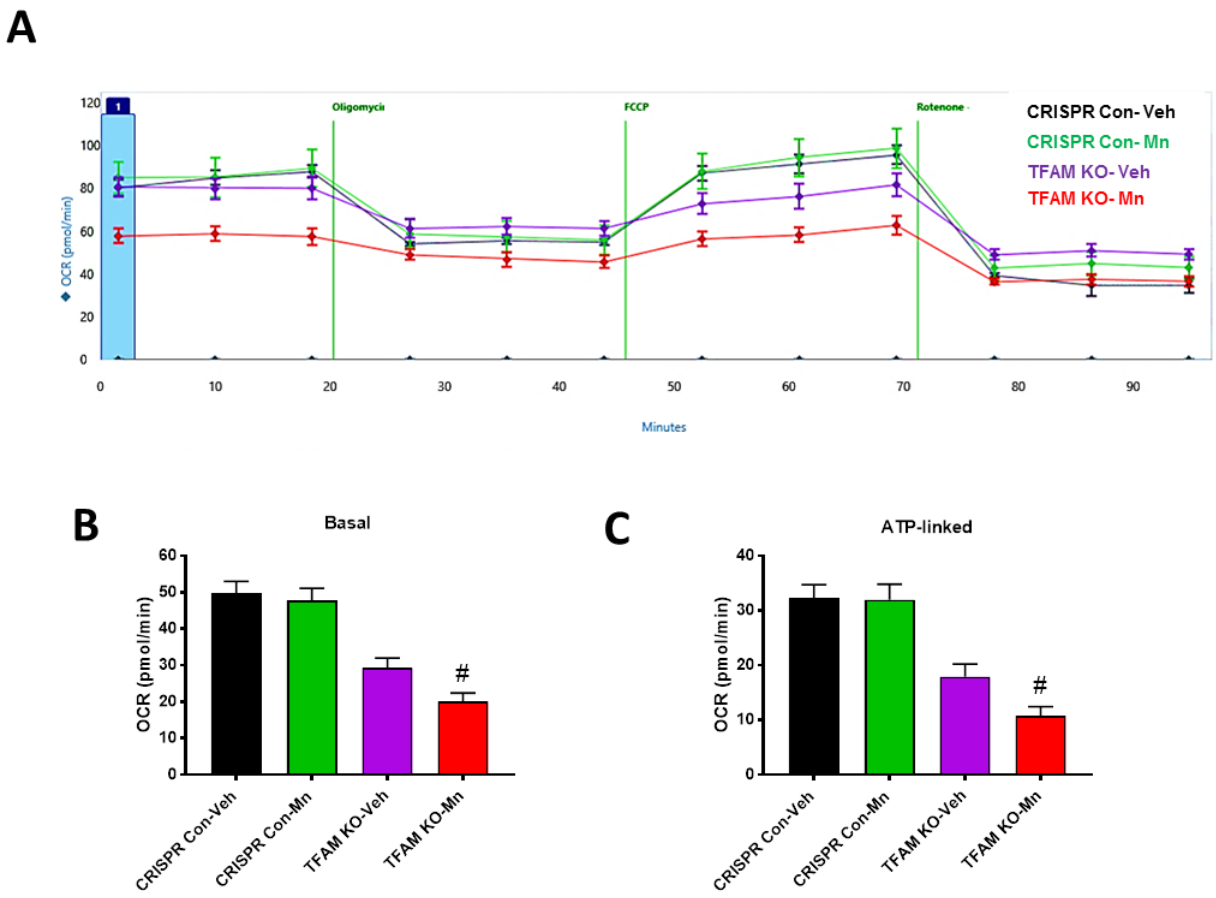

Figure 7. Mn exposure potentiated mitochondrial deficits in TFAM-KO neuronal cells

A, CRISPR/Cas9-based TFAM-KO and Control N27 cells were treated with or without 100 µM Mn for 24 h and mitochondrial dynamics were measured using Seahorse XF24 analyzer. *A*, Quantification of basal respiration rate prior to MitoStressor injections. *B*, Quantification of ATP-linked respiration following oligomycin injection. Graphical results represented as the mean±SEM (n=4-5/group). #, $p<0.05$, versus untreated control from same cell type.

Table 1. Summary of Bonferroni post-tests from repeated measures ANOVA for various behavioral parameters. ns, not significant; *, p<0.05; **, p<0.01; ***, p<0.001.

Horizontal Activity	**8**	**9**	**10**	**11**	**12 wk**
C57-Control vs. C57-Mn	ns	ns	ns	ns	ns
C57-Control vs. MitoPark-Control	ns	*	ns	ns	ns
C57-Control vs. MitoPark-Mn	ns	ns	ns	ns	***
MitoPark-Control vs. MitoPark-Mn	ns	ns	ns	ns	ns
Vertical Activity	**8**	**9**	**10**	**11**	**12 wk**
C57-Control vs. C57-Mn	ns	ns	ns	ns	ns
C57-Control vs. MitoPark-Control	ns	ns	ns	ns	ns
C57-Control vs. MitoPark-Mn	ns	ns	ns	*	**
MitoPark-Control vs. MitoPark-Mn	ns	*	ns	ns	ns
RotaRod	**8**	**9**	**10**	**11**	**12 wk**
C57-Control vs. C57-Mn	ns	ns	ns	ns	ns
C57-Control vs. MitoPark-Control	ns	ns	ns	ns	ns
C57-Control vs. MitoPark-Mn	ns	ns	**	***	***
MitoPark-Control vs. MitoPark-Mn	ns	ns	**	***	***
Total Distance Traveled	**8**	**9**	**10**	**11**	**12 wk**
C57-Control vs. C57-Mn	ns	ns	ns	ns	ns
C57-Control vs. MitoPark-Control	ns	ns	ns	ns	ns
C57-Control vs. MitoPark-Mn	ns	ns	ns	ns	ns
MitoPark-Control vs. MitoPark-Mn	ns	ns	ns	ns	ns
Rearing Activity	**8**	**9**	**10**	**11**	**12 wk**
C57-Control vs. C57-Mn	ns	ns	ns	ns	ns
C57-Control vs. MitoPark-Control	ns	ns	ns	ns	ns
C57-Control vs. MitoPark-Mn	ns	ns	ns	*	**
MitoPark-Control vs. MitoPark-Mn	ns	ns	ns	ns	ns

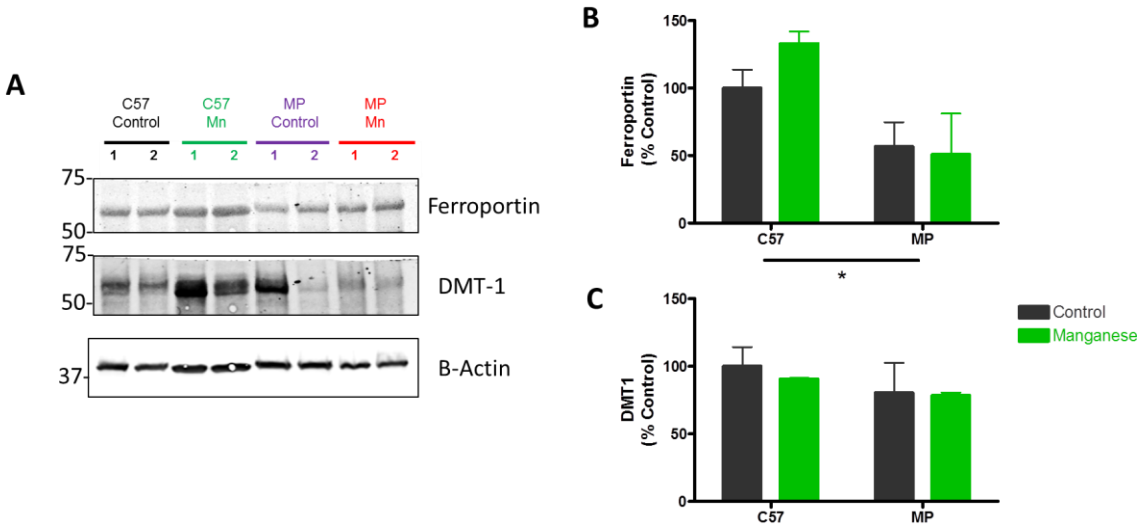

Figure S1. Expression of manganese transporters in substantia nigra tissues from littermate control and MitoPark mice treated with manganese. A, Representative western blots from substantia nigra tissues. B, densitometric analysis showing significant decrease in ferroportin in MitoPark mice (MP) when compared to their littermate controls (C57) by two-way ANOVA. No significant treatment effect was observed upon treatment with 10mg/kg manganese by oral gavage for 30 days. C, no significant treatment or genotype effect on DMT-1 expression was observed.

CHAPTER 4

MITO-APOCYNIN PREVENTS MITOCHONDRIAL DYSFUNCTION, MICROGLIAL ACTIVATION, OXIDATIVE DAMAGE AND PROGRESSIVE NEURODEGENERATION IN MITOPARK TRANSGENIC MICE

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Monica Langley[1], Anamitra Ghosh[1], Adhithiya Charli[1], Souvarish Sarkar[1], Muhammet Ay[1], Jie Luo[1], Jacek Zielonka[2], Timothy Brenza[3], Brian Bennett[4], Huajun Jin[1], Shivani Ghaisas[1], Benjamin Schlichtmann[3], Dongsuk Kim[1], Vellareddy Anantharam[1], Arthi Kanthasamy[1], Balaji Narasimhan[3], Kalyanaraman Balaraman [2], Anumantha G. Kanthasamy[1]*

[1]Department of Biomedical Sciences, Iowa Center for Advanced Neurotoxicology, Iowa State University, Ames, IA 50011, USA

[2]Department of Biophysics, Medical College of Wisconsin, Milwaukee, WI 53226, USA

[3]Department of Chemical and Biological Engineering, Iowa State University, Ames, IA 50011, USA

[4]Department of Physics, Marquette University, Milwaukee, WI 53233, USA

***Correspondence**: Anumantha Kanthasamy, Ph.D. Distinguished Professor and Chair, Department of Biomedical Sciences, Iowa State University, Ames, IA 50011

Telephone: (515) 294-2516; Fax: (515) 294-2315, Email: akanthas@iastate.edu

Abstract

Aims: Parkinson's disease (PD) is a neurodegenerative disorder characterized by progressive motor deficits and degeneration of dopaminergic neurons. Caused by a number of genetic and environmental factors, mitochondrial dysfunction and oxidative stress play a role in neurodegeneration in PD. By selectively knocking out mitochondrial transcription factor TFAM in dopaminergic neurons, the transgenic MitoPark mice recapitulate many signature features of the disease, including progressive motor deficits, neuronal loss, and protein inclusions. In the present study, we evaluated the neuroprotective efficacy of a novel mitochondrially-targeted antioxidant, mito-apocynin, in MitoPark mice and cell culture models of neuroinflammation and mitochondrial dysfunction.

Results: Oral administration of Mito-apocynin (10 mg/kg, thrice a week) showed excellent CNS bioavailability and significantly improved locomotor activity and coordination in MitoPark mice. Importantly, Mito-apocynin also partially attenuated severe nigrostriatal degeneration in MitoPark mice. Mechanistic studies revealed that Mito-apo improves mitochondrial function and inhibits NOX2 activation, oxidative damage and neuroinflammation.

Innovation: The properties of mito-apocynin identified in the MitoPark transgenic mouse model strongly support potential clinical applications for Mito-apocynin as a viable neuroprotective and anti-neuroinflammatory drug for treating PD when compared to conventional therapeutic approaches.

Conclusion: Collectively, our data demonstrate, for the first time, a novel orally active apocynin derivative improves behavioral, inflammatory, and neurodegenerative processes in a severe progressive dopaminergic neurodegenerative model of PD.

Introduction

Parkinson's disease (PD) is a debilitating neurodegenerative disorder, affecting millions of people worldwide, and is triggered by a complex interplay of genetic and environmental factors. PD neuropathology is characterized by a loss of dopaminergic neurons in the substantia nigra (SN), loss of DA in the striatum, and accumulation of abnormal α-synuclein protein, a major constituent of Lewy bodies and Lewy neurites (63). Motor symptoms such as bradykinesia, tremor, rigidity, and postural instability are still used to clinically diagnose PD. Although it is known that neuronal loss leads to DA deficiency and motor dysfunction, less is understood regarding the mechanisms underlying the loss of SN neurons (55). Neuroinflammation, oxidative stress and mitochondrial dysfunction have all been implicated in PD pathogenesis (65,68). Although mitochondrial dysfunction is well established as an overriding pathophysiological indication of PD, no effective treatment options are available to improve the efficiency of mitochondrial function in PD.

Mitochondrial toxin-based PD models such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 6-hydroxydopamine (6-OHDA) and rotenone are used to screen compounds for further development. However, toxin-based PD models do not reflect human PD mainly due to acute nature of neuronal injury, toxicant-drug interactions and between-subject response variation (6,21,47). MitoPark mice were created by selective inactivation of mitochondrial transcription factor A (TFAM) in the nigrostriatal pathway (22). Key pathological features of PD such as adult onset, progressive neurodegeneration, protein inclusions, and progressive motor deficits ameliorated by L-DOPA have been identified in MitoPark mice (27), making the model ideal for testing novel mitochondria-targeted neuroprotective agents.

A strong therapeutic potential exists for mitochondria-targeted antioxidants (MTAs) in neurodegenerative disorders (39). We recently demonstrated that apocynin (4-hydroxy-3-methoxyacetophenone), a plant-derived molecule structurally related to vanillin, and its dimer, diapocynin, are neuroprotective in the MPTP and LRRK2^{R1441G} mouse models (18,32). As an inhibitor of NOX2 activity, apocynin has been studied in various cell culture and animal models of PD (1,12,28). Although the high doses of apocynin and diapocynin (300 mg/kg) used in these animal studies were well tolerated, more efficacious apocynin analogs are needed. We therefore synthesized a series of mitochondria-targeted apocynins (conjugated to a triphenylphosphonium cation moiety via an alkyl chain with differing chain lengths (C_2-C_{11} carbon atoms)). Using Mito-apo-C_2, we have recently established that Mito-apo protected against neuroinflammation and neurodegeneration in the MPTP model (33). Herein, we evaluated the disease-modifying efficacy of a long acting Mito-apo-C_{11} in the MitoPark model that recapitulates key features of PD including mitochondrial dysfunction, microglial activation, and dopaminergic degeneration. Our results show that oral administration of Mito-apo dampens chronic, progressive behavioral deficits and dopaminergic neurodegeneration. Additional mechanistic studies support the enhanced mitochondrial function and anti-inflammatory properties of Mito-apo.

Results

Mito-apo restores mitochondrial function in dopaminergic neuronal cells

We first assessed the effect of Mito-apo on key markers of mitochondrial bioenergetics of dopaminergic neuronal cells. The Seahorse extracellular flux analyzer revealed the basal respiration rate to be nearly double the control OCR values in 10- to 30-µM Mito-apo-treated N27 dopaminergic neuronal cells (Fig. 1A-B). Upon treatment with oligomycin, Mito-apo-treated cells showed a significantly higher ATP-linked respiration (Fig. 1A, C) and respiratory capacity (Fig. 1A, D) than untreated cells. As expected, in the positive control, 1 µM rotenone abolished the OCR signal (Fig. 1A-D).

Given that knockdown (KD) of TFAM has been shown to result in cellular ATP depletion and loss of mitochondrial function in mammalian cell culture studies (48), we determined if Mito-apo could improve mitochondrial function in stable TFAM-KD N27 cells that we generated using the CRISPR/Cas9 system as described in supplementary materials. Transduction of N27 cells with TFAM-KD resulted in >80% loss in TFAM mRNA levels relative to control cells (Fig. S1). In contrast to control N27 cells, TFAM-KD cells had significantly lower cellular ATP levels (Fig. 1E). 1 µM rotenone for 3 h (positive control) dramatically decreased cellular ATP levels in control N27 cells. Mito-apo did not significantly alter the ATP levels in control cells. However, exposure to 10-30 µM Mito-apo for 3 h dramatically increased cellular ATP levels in TFAM-KD N27 cells, suggesting that Mito-apo replenishes ATP levels in mitochondrially stressed cells.

Aconitases are iron-sulfur-containing enzymes that isomerize citrate and are inactivated during mitochondrial oxidative stress. Measurement of aconitase enzyme activity in TFAM-KD cells revealed significantly decreased *m*-aconitase activity when compared to

control N27 cells, indicating that TFAM-KD led to significant mitochondrial oxidative damage (Fig. 1F). However, treating the TFAM-KD cells with 10-30 µM Mito-apo for 3 h returned *m*-aconitase activity to levels present in control N27 cells. Mito-apo did not significantly alter the *m*-aconitase activity in control cells, and rotenone strongly reduced *m*-aconitase activity in both cells (Fig. 1F).

We also assessed loss of mitochondrial function with MitoSOX™ red dye in TFAM-KD and control N27 cells. Basal oxidation of MitoSOX probe was significantly higher in TFAM-KD than in control cells (Fig. 1G). A 12-h exposure to 10 and 30 µM Mito-apo decreased the rate of probe oxidation in TFAM-KD, but not in control N27 cells (Fig. 1G). As observed previously using hydroethidine probe in control N27 cells (20), rotenone strongly stimulated MitoSOX oxidation in both cell lines.

MitoTracker red dye was used to visualize integrity or structural abnormalities in TFAM-KD N27 cells. Healthy mitochondria, such as those from control cells, appear as long, thread-like structures (Fig. 1H). Relative to those of control cells, the mitochondria of TFAM-KD N27 cells were significantly shorter (Fig. 1I) and more circular (Fig. 1J), further indicating a striking loss of mitochondrial structural integrity. Although 10 µM Mito-apo was not enough to improve mitochondrial morphology, TFAM-KD cells treated with 30 µM Mito-apo exhibited significantly increased mitochondrial length (Fig. 1H, I). Collectively, results from these experiments suggest that Mito-apo restores mitochondrial morphology and function in TFAM-KD N27 cells.

Mito-apo stalls progression of motor deficits

Considering that Mito-apo was capable of replenishing mitochondrial function in TFAM-KD N27 cells, we decided to determine if Mito-apo is neuroprotective in the MitoPark mouse of PD in which TFAM is selectively inactivated in dopaminergic neurons. Preceded by early neurochemical alterations, these mice exhibit behavioral deficits by 12 wks, which become progressively worse by 24 wks (34). Our dose for this study was initially based on the severity of our model and the therapeutic efficacy reported for other model systems in previous publications (19,33). Given that MitoPark is one of the most severe models of PD, we considered that the lower dose of mito-apocynin used in the MPTP paper (3 mg/kg/day) and LRRK2 paper (3 mg/kg, 3x/week) might not be sufficient in the MitoPark mice, and therefore used a higher dose. As depicted in our treatment paradigm (Fig. 2A), MitoPark and age-matched C57 mice were orally administered either Mito-apo (10 mg/kg, 3X/wk) or vehicle (10% ethanol/PBS) beginning at age 13 wks. Open-field activity and RotaRod behavioral performances were evaluated at ages 18, 21 and 24 wks (Fig. S5).

Decreased movement, as expected in 24 wks MitoPark mice, is shown by representative Versaplot maps (Fig. 2B) and improved locomotion can be seen in plots of the Mito-apo-treated MitoPark mice. Consistently, 24-wk MitoPark mice exhibited decreased horizontal activity (Fig. 2C), vertical activity (Fig. 2D), total distance traveled (Fig. 2E), and time spent on the RotaRod (Fig. 2F). Mito-apo treatment did not have any significant effect on any of these behavioral parameters in age-matched control mice, but importantly, the horizontal and vertical activity levels and RotaRod performance of MitoPark mice were significantly retained by Mito-apo treatment in contrast to vehicle-treated MitoPark mice.

Mito-apo treatment dampens nigrostriatal TH neuronal loss and striatal DA depletion

Neurodegeneration in the SN is progressive and severe in aged MitoPark mice (27). Brain sections from 24-wk MitoPark mice were immunostained for TH to detect dopaminergic neurons. A significant loss of TH^+ cell bodies and terminals in the SN and striatum, respectively, occurred in vehicle-treated 24-wk MitoPark mice (middle panel) in contrast to vehicle-treated age-matched C57 mice (top panel) (Fig. 3A). DAB immunostaining showed significantly higher levels of TH immunoreactivity (Fig. 3A) and neuronal counts (Fig. 3B) in the striatum and SN of Mito-apo-treated MitoPark mice compared to vehicle-treated MitoPark mice. Mito-apo did not alter the number of TH neurons in control mice. Further confirmation came from Western blots and their densitometric analysis, where higher levels of TH protein expression were observed in the SN of Mito-apo-treated MitoPark mice versus vehicle-treated MitoPark mice (Fig. 3C).

Since Mito-apo-treated MitoPark mice had more dopaminergic neurons remaining in the SN, we performed HPLC with electrochemical detection to determine if striatal levels of DA and its metabolites were also protected. We observed a severe loss of DA, DOPAC and HVA, respectively, in the striatum of vehicle-treated MitoPark mice compared to age-matched control mice (Fig. 3D-F). In contrast, Mito-apo increased DA and DOPAC by 2.6- and 2.0-fold, respectively, when compared to vehicle-treated MitoPark mice. Mito-apo did not affect striatal DA and its metabolite levels in C57 black mice. Together, these data suggest that Mito-apo substantially increases the TH neuronal cell count and striatal DA levels in aged MitoPark mice.

Mito-apo attenuates mitochondrial aconitase inactivation and oxidative damage

To further determine the effect of Mito-apo on mitochondrial redox status in the MitoPark model, we used *ex vivo* EPR analysis to see if Mito-apo protects against inactivation of mitochondrial aconitase. Previously, we had shown that Mito-Q_{10} treatment mitigates MPTP-mediated inactivation of mitochondrial aconitase and inhibits the increase in the EPR signal intensity at $g' = 6$ (attributed to cytochrome c) detected in brain tissues obtained from MPTP-treated mice (31). Similarly, in this study we show that the EPR spectra in the $g \approx 2$ region (blue bracket, Fig. 4A) from MitoPark striatal samples (middle spectra, Fig 4B) have higher fractional intensities, which are primarily due to the inactive, oxidized $[3Fe\text{-}4S]^{+}$ cluster of aconitase. EPR spectral signals were internally normalized to high spin (H.S.) heme (blue arrow, Fig. 4A and C). A similar trend occurred in cortical tissues, but was insignificant (Fig. S2). Most importantly, Mito-apo treatment significantly reduced the fractional intensities from the $[3Fe\text{-}4S]^{+}$ cluster signal in MitoPark mice relative to vehicle treatment (Fig. 4D).

Oxidative damage biomarkers such as nitrotyrosine-containing proteins and lipid peroxidation product 4-HNE have been shown in animal models of PD and have high correlation to disease states (32,51). Confocal imaging of IHC double-labeled with TH antibody and Western blot analysis revealed increased 4-HNE expression in the SN of aged MitoPark mice but not in control mice (Fig. 4E-F). Mito-apo administration almost completely suppressed 4-HNE levels in the SN of aged MitoPark mice. These results demonstrate that Mito-apo attenuates mitochondrial aconitase inactivation in the striatum and oxidative damage in nigral neurons of aged MitoPark mice.

Mito-apo inhibits microglia activation in the SN

Neuroinflammation is now recognized to play a key role in dopaminergic neurodegeneration in human PD patients and in mouse models (17,53,66). Although mitochondrial dysfunction and oxidative stress act as key players in the progressive degenerative MitoPark mouse model, a presence of neuroinflammation in this model has not yet been explored. Western blotting and DAB immunostaining show that IBA1 expression increases in the SN of MitoPark mice (Fig. 5A-B). However, levels of the IBA1 protein were significantly lower in Mito-apo-treated MitoPark mice. IBA1 DAB immunostaining (Fig. 5B) revealed an increased number of microglia in the aged, vehicle-treated MitoPark SN, and at higher magnification, increased soma size and fewer processes, indicating an activated phenotype. These features occurred to a much lesser extent in the Mito-apo-treated MitoPark mice. Indeed, the cell count per field (Fig. 5C) was significantly lower in the Mito-apo-treated group, average soma size was smaller (Fig. 5D), and ImageJ skeleton analysis revealed more process end points per cell in Mito-apo-treated MitoPark mice when compared to vehicle-treated MitoPark mice (Fig. 5E). Other variables indicative of a normal microglia phenotype, such as the number of branches, number of junctions, and the longest shortest path, were significantly reduced in the MitoPark group when compared to controls (Fig. 5F-H). However, the MitoPark mice treated with Mito-apo did not exhibit significant changes in these microglial morphological changes when compared to age-matched control mice. We also observed $CD68^{+}$ staining, a marker of microglia activation, in vehicle-treated MitoPark mice that was not present in control or Mito-apo-treated MitoPark mice (Fig. S7).

Mito-apo decreases NOX2 and iNOS activity in microglia

An increase in NOX2 and iNOS expression during microglial activation may synergistically contribute to neuronal death (46). Since Mito-apo treatment inhibits microglia activation, we then tested whether it also dampened NOX2 activity and iNOS induction. Immortalized microglial cells were generated as described in the Methods and obtained as a kind gift from Dr. Douglas Golenbock for conducting the following experiments (37). To better define the therapeutic target of Mito-apo in microglia, we examined NOX2 activation by $p47^{phox}$ translocation from cytosol to the membrane. Western blot analysis revealed that LPS robustly increased the amount of membrane-bound $p47^{phox}$ in microglia, while the cytosolic $p47^{phox}$ was significantly reduced when compared to control cells (Fig. 6A). Furthermore, this is substantiated by immunocytochemical results showing increased co-localization of $p47^{phox}$ with cholera toxin subunit B (CTXB-555), a membrane marker, following LPS treatment (Fig. 6B). LPS-stimulated microglia co-treated with Mito-apo showed a significant decrease in membrane $p47^{phox}$ and an increase in cytosolic $p47^{phox}$ when compared to vehicle-treated LPS-stimulated microglia (Fig. 6A). Similarly, reduced co-localization of $p47^{phox}$ and CTXB-555 was observed in Mito-apo-treated LPS-stimulated microglia (Fig. 6B). Together, these data suggest that Mito-apo can effectively prevent $p47^{phox}$ membrane recruitment, which is otherwise obligatory for NOX2 activation.

Our previous publication shows that HL60 cells differentiated into neutrophils and treated with phorbol myristate acetate (PMA) can be used as a reliable source of endogenous NOX2 activation (73). To test the effect of Mito-apo treatment on NOX2 activity in intact cells, we used all-trans retinoic acid (ATRA)-differentiated HL60 cells, stimulated with PMA. We utilized hydropropidine (HPr^+) as the probe for NOX2-derived superoxide radical

anion ($O_2^{\bullet-}$) and coumarin boronic acid (CBA) as a probe for hydrogen peroxide (H_2O_2) (73,75). The 30-min pretreatment of cells with a low micromolar concentration (<10 µM) of Mito-apo led to a dose-dependent inhibition of the PMA-stimulated generation of $O_2^{\bullet-}$ and H_2O_2 (Fig. 6C). This demonstrates that Mito-apo directly or indirectly mitigates NOX2 activity.

Next, we tested the effect of Mito-apo on oxidant production in microglial cells. After exposing microglial cells to LPS (1 µg/ml) for 12 h in the presence or absence of Mito-apo (10-30 µM), Griess assay revealed that Mito-apo completely blocked LPS-induced nitric oxide production (Fig. 6D). In addition, LC-MS-based monitoring of the conversion of the *o*-MitoPhB$(OH)_2$ probe into the phenolic product (*o*-MitoPhOH) (10,11,74,75) indicates significant suppression of mitochondrial oxidant levels (Figure 6E). The Mito-apo co-treatment also significantly attenuated LPS-induced increases in the levels of supernatant cytokines, including TNFα, IL-1β, IL-12 and IL-6, as measured by Luminex multiplex immunoassay (Fig. 6F). For comparison, 10 µM Vitamin C and 10 µM apocynin were also tested, but neither treatment significantly attenuated LPS-stimulated neuroinflammation endpoints at this dose (Fig. S6).

Mito-apo attenuates iNOS and NOX2 expression in the substantia nigra of MitoPark mice

Consistent with our *in vitro* results, double immunolabeling experiments confirmed the increased iNOS expression occurred in $IBA1^+$ microglia and was significantly reduced in Mito-apo-treated MitoPark mice (Fig. 7A). Western blotting revealed an increase in iNOS expression in the SN of 24-wk MitoPark mice that was significantly attenuated in the Mito-apo-treated mice (Fig. 7B). In addition, $gp91^{phox}$, a subunit of NOX2, was upregulated in

microglia of the SN of aged MitoPark mice (Fig. 7C-D), yet was significantly reduced in the Mito-apo-treated group, as evidenced by Western blotting and IHC double-labelling with IBA1.

Given that NOX4 isoform has been shown to localize in the mitochondria of neurons (7), we also assessed whether Mito-apo alters NOX4 in our mitochondrially defective cell and animal models of PD. In TFAM-KD N27 dopaminergic cells, NOX4 levels increased significantly when compared to control cells (Fig. S4A). However, Mito-apo treatment did not alter NOX4 levels in TFAM-KD cells. Furthermore, NOX4 mRNA and protein levels were not altered in the SN of either MitoPark mice or MitoParks treated with Mito-Apo when compared to age-matched control mice (Fig. S4B-C). These data suggest Mito-apo is more selective for NOX2 over NOX4 in neuronal cells and NOX4 is not a major contributor to oxidative damage in the MitoPark model.

Peroxynitrite, formed when superoxide reacts with nitric oxide, nitrates tyrosine residues on proteins and contributes to neuronal cell death (33,75). Increased nitrative modification was evidenced by higher 3-NT expression in the SN of 24-wk MP mice compared to C57 black mice (Fig. 7E). Notably, 3-NT expression was significantly suppressed in Mito-apo-treated MP mice. Overall, these data show that Mito-apo attenuated microglia activation and neuronal cell death in MitoPark mice by suppressing oxidative and nitrative stress.

Mito-apo is CNS bioavailable without systemic adverse effects

Finally, we determined if Mito-apo reaches its target brain tissues, the SN and striatum. Mice were orally administered Mito-apo (10 mg/kg), sacrificed, and tissues were harvested at 30 min, 3, 6, 12, 24 and 48 h. Striatal and nigral regions were processed for

HPLC. Here, we have provided evidence that Mito-apo reaches its target brain tissues, the SN and striatum, and did not cause systemic adverse effects (Figure S3 and Tables 1 and 2). We also conducted a dose response study at the 6-h time point, when Mito-apo availability peaks in brain tissues (Fig. S3), yet did not detect any significant difference between 10 and 30 mg/kg Mito-apo oral administration.

Clinical pathology can be particularly useful in predicting toxicity in animal models for pre-clinical evaluation (5,59). Blood plasma, collected by cardiac puncture at 3 h and 48 h after gavaging mice with 10 mg/kg Mito-apo, was analyzed using the Abaxis system. Clinical pathology variables are reported in Tables 1 and 2. At 3 h, the only significant difference between vehicle and Mito-apo-treated C57 black mice was blood glucose level (Table 1). As mice were not fasted, the range of values for both groups are considered acceptable (57). Alkaline phosphatase (ALP) levels, an indicator of hepatobiliary or bone disease, were significantly less in Mito-apo-treated mice than in controls after 48 h (Table 2), but still within normal reference intervals. Calcium and phosphorous levels were elevated in the Mito-apo-treated group, but no other significant changes were observed at 48 h post-gavage. These results suggest that Mito-apo can rapidly cross the blood brain barrier and persist in the CNS to produce its neuroprotective effect without contributing any major acute adverse effects.

Discussion

Oxidative stress, mitochondrial dysfunction and neuroinflammation contribute to dopaminergic degeneration in PD (3,13,66). ROS derived from NOX2 and mitochondria potentiate neurodegeneration through oxidative stress and promote pro-inflammatory events (12,56). In this study, we tested a recently created pharmacophore, Mito-apo, in cell culture and animal models of mitochondrial dysfunction and neuroinflammation. First, Mito-apo treatment increased OCR in a dopaminergic cell line, indicating a bioenergetic effect. This was further clarified by showing that treatment with Mito-apo increased aconitase activity and ATP levels, improved mitochondrial structural integrity and decreased mitochondrial oxidants in TFAM-KD N27 cells. In MitoPark mice, Mito-apo treatment attenuated progressive motor deficits, striatal neurotransmitter depletion and nigrostriatal degeneration, suggesting a neuroprotective property. Mito-apo treatment also prevented microglial activation, pro-inflammatory mediators, NOX2 and iNOS activation, and oxidative stress marker formation. Thus, our data demonstrate anti-inflammatory, anti-oxidant and neuroprotective properties of a new apocynin derivative, Mito-apo, in MitoPark mice that possess pathological features of chronic dopaminergic neurodegeneration including mitochondrial dysfunction, oxidative damage, and neuroinflammation.

Mitochondrial dysfunction can be found in toxin-based models, such as the MPTP and 6-OHDA treatment paradigms, and in genetic models, such as the LRRK2- and PINK1-based models (3,16,44,62,71). Wang et al. (69) demonstrated that TFAM inactivation results in respiratory chain deficiency and increased *in vivo* apoptosis, and proposed that the resulting loss of mitochondrial function has important therapeutic implications for human disease. Although the MitoPark model, with TFAM knocked out in the nigrostriatal pathway,

does not mirror any specific genetic mutation associated with PD, both TFAM and mitochondrial DNA changes have been implicated in aging and PD (2,29,30,54). Similarly, the TFAM-KD N27 dopaminergic cells generated using the CRISPR/Cas9 system manifested mitochondrial dysfunction as reduced *m*-aconitase activity and ATP levels, increased oxidant production, and altered mitochondrial morphology. Our results clearly indicate that Mito-apo restores functional mitochondria by increasing mitochondrial bioenergetics and inhibiting production of oxidants. Thus, treatment with a MTA can alleviate the loss of mitochondrial structure and function in a cell culture model of mitochondrial dysfunction.

We next determined if Mito-apo exerts its protective action on mitochondrial redox *in vivo*. To gain insights into any effect of Mito-apo on oxidative stress amelioration in MitoPark mice, we monitored the EPR signals due to mitochondrial aconitase iron–sulfur clusters in brain tissue at cryogenic temperatures. ROS-induced oxidation of the catalytically-required $[4Fe–4S]^{2+}$ cluster inactivates the aconitase enzyme due to loss of the labile iron ion, forming the EPR-detectable $[3Fe–4S]^{+}$ cluster. Mito-apo treatment significantly inhibited the development of the $[3Fe–4S]^{+}$ cluster EPR signal from mitochondrial aconitase in MitoPark mice. These results suggest that Mito-apo treatment can effectively block *m*-aconitase inactivation in aged MitoPark mice. Advancing age is a main risk factor in neurodegenerative disease and is correlated with increased mitochondrial dysfunction and oxidative stress, which leads to irreversible structural changes in proteins, lipids, and DNA (51,65,68). Here we found that levels of 4-HNE-modified proteins, an unsaturated aldehyde generated during lipid peroxidation and marker of oxidative damage, were significantly higher in the SN of 24-wk MitoPark brains compared to age-matched C57 black controls, yet Mito-apo treatment significantly decreased these levels. Hence, oxidative stress associated with the inherent

mitochondrial dysfunction in the MitoPark model can be reduced by long-term, low-dose treatment with Mito-apo.

In the CNS, microglial activation and the production of pro-inflammatory factors including cytokines, ROS and RNS are hallmarks of inflammatory reactions. Our IHC and Western blot analyses revealed significantly increased IBA1 expression in the SN of 24-wk MitoPark mice coupled with increased soma size and fewer processes, suggesting increased microglia activation in 24-wk MitoPark mice. Mito-apo-treated MitoPark mice had significantly fewer $IBA1^{+}$ cells, decreased soma size, and increased branching complexity consistent with ramified microglia when compared to vehicle-treated MitoPark mice. Mito-apo treatment also effectively decreased iNOS and $gp91^{phox}$ protein levels in the $IBA1^{+}$ nigral cells of MitoPark mice. Activation of NOX2 and mitochondrial ROS was proposed to involve crosstalk that increases production of both in a feed-forward cycle (15,46). NOXs play an obvious role in dopaminergic neurodegeneration via ROS generation (9,52). However, their involvement in PD is potentially more complex, involving other contributing factors such as protein aggregation and mitochondrial dysfunction (13,15,20). In animal models of PD in which proinflammatory enzymes are elevated, anti-inflammatory therapies have successfully modulated the neurodegenerative process (32,56). Reactive microgliosis results from stressed neurons secreting factors that activate microglia, beginning the cycle of neuroinflammation and neuronal damage underlying neurodegenerative diseases (4,42,45). Although neuroinflammation was not originally defined in the MitoPark model, we show here that reactive microgliosis is likely involved, implicating an important therapeutic potential for MTA and anti-inflammatory drugs in PD. This finding is salient considering that

chronic neuroinflammation is now perceived to mediate PD pathogenesis and current drugs only provide symptomatic relief (60,66).

By restricting $p47^{phox}$ from translocating to the membrane and subsequently inhibiting superoxide production by NOX2 in microglia, Mito-apo reduced inflammation in addition to its role as an antioxidant in neurons. This combined effect on both microglia and neurons may help to break up a self-propelling cycle of neuroinflammation and neurodegeneration characteristic of PD. This approach was also recently found to be effective in the toxin-based MPTP model (28). It should be noted that the severity of the MitoPark model allowed for only a modest recovery of motor function with Mito-apo treatment due to the complex nature of the neurodegenerative process. We explored the potential benefit of a higher dose by performing a dose-response study at the time point with the highest concentration of Mito-apo in the brain (Fig. S3D). However, our results did not show significantly more Mito-apo detectable in brain tissues of the 30 mg/kg group versus 10 mg/kg. This suggests that even at a higher dose, Mito-apo would not have provided an additional benefit. Although oxidative stress and neuroinflammation are key players in the cell death mechanisms leading to PD, other mechanisms such as protein aggregation, ubiquitin-proteasome system dysfunction, mitophagy, and inflammasome-dependent neuroinflammation have not yet been fully addressed in this model or with this compound. Furthermore, our group and others have shown that targeting a single pathophysiological pathway is not typically enough to completely restore function in the MitoPark model (24,41). These observations warrant future studies into the therapeutic role of Mito-apo and the development of combination therapy modalities for PD.

Recently, we have demonstrated that Mito-apo prevents hyposmia and motor deficits in the LRRK2 mouse model (19). However, the effect of Mito-apo on mitochondrial function, microglial activation, NOX2 activation, and dopaminergic degeneration was not directly investigated in that model. Although circadian and cognitive changes occur in MitoPark mice (25,43), the nonmotor Parkinsonian phenotype of these mice still needs to be fully characterized. Moreover, Mito-apo's efficacy in treating nonmotor symptoms in PD animal models should be evaluated since antioxidants have been shown to successfully improve cognitive and neuropsychiatric symptoms in various neurodegenerative models (64,67).

Based on our findings using a progressively degenerating and mitochondrially dysfunctional animal model of PD, Mito-apo may outperform other experimental drugs by being well tolerated yet bioavailable in the brain when delivered orally, and exhibiting potent anti-neuroinflammatory and anti-oxidant effects at a low dose. Here, we have demonstrated that Mito-apo protected dopaminergic neurons, and reduced oxidative and nitrative stress, glial cell activation and inflammatory reactions. NOX2-mediated superoxide production is thought to be essential for maintaining chronic neuroinflammation which potentiates the neurodegenerative process through oxidative stress (9,52). NOX2 knockout or treatment with NOX2 inhibitors in cell culture and mice studies suggest that inhibiting NOX2 breaks the vicious, self-propelling cycle of reactive microgliosis, making it a valuable target for PD therapy (61,70). These properties strongly support potential clinical applications for Mito-apo as a viable neuroprotective and anti-neuroinflammatory drug for treating PD when compared to conventional therapeutic approaches which only target downstream consequences of microglia activation and inflammation-mediated oxidative stress. Overall, we demonstrate

that Mito-apo treatment effectively attenuates progressive motor deficits, neuroinflammation, neurochemical depletion and loss of nigral TH neurons, revealing a neuroprotective effect in a comprehensive PD model that recapitulates salient features of chronic progressive dopaminergic neurodegeneration. Our proof of principle preclinical study warrants further translation of Mito-apo to preclinical safety evaluations and to subsequent clinical testing.

Innovation

Advances in drug discovery for neurodegenerative diseases have been hampered by the lack of models that recapitulate the chronic, progressive neurodegeneration characteristic of PD. However, a recently developed genetic model of mitochondrial dysfunction, known as MitoPark mice, mirrors many key features of PD. Recently, we developed a new class of pharmacological agents to effectively dampen the major pathophysiological processes associated with PD (19). The properties of mito-apocynin identified in this model strongly support potential clinical applications for mito-apocynin as a viable neuroprotective and anti-neuroinflammatory drug for treating PD when compared to conventional therapeutic approaches.

Materials and Methods

Chemicals

Rotenone (R8875), oligomycin (75351), antimycin A (A8674), dopamine hydrochloride (H8502), 3-4-dihydroxyphenylacetic acid (DOPAC, 850217), homovanillic acid (HVA, H1252), 3,3'-diaminobenzidine (DAB, D5637), Griess reagent (4410), puromycin (P8833) and hydrogen peroxide (H325) were all purchased from Sigma (St Louis, MO). The CellTiter-Glo Cell Viability assay kit (G5421) was bought from Promega (Madison, WI). We purchased RPMI 1640 (11875093) and DMEM (11330) media, fetal bovine serum (FBS, 26140), L-glutamine (25030081), penicillin-streptomycin (15140122), MitoTracker red (M-7512), and MitoSox (M36008) from Invitrogen (Carlsbad, CA) and the Seahorse Flux Pak calibration solution and FCCP (103015) from Seahorse Biosciences (Billerica, MA). The aconitase assay kit (ab83459), anti-4-HNE (mab3249), anti-IBA1 (ab5076), and gp91phox (ab129068) antibodies were purchased from Abcam (Cambridge, MA). Anti-NOS2 (sc-650) and $p47^{phox}$ (H-195) antibodies were obtained from Santa Cruz Biotech (Dallas, TX), anti-IBA1 antibody (019-19741) was purchased from Wako (Richmond, VA), and anti-TH (mab318) and anti-3-NT (06-284) antibodies were purchased from Millipore (Billerica, MA).

Mito-apocynin Synthesis

Mito-apo-C2 and Mito-apo-C11 were synthesized as described previously (19). Briefly, acetylvanillic acid and thionyl chloride were mixed to form acetylvanillic acid chloride which was dissolved in dichloromethane and aminoalkyltriphenylphosphonium

bromide and pyridine. The solution was purified on a silica gel column and the acetyl group was removed by hydrolysis.

Cell Culture and CRISPR/Cas-based Knockout of TFAM in N27 Cells

For *in vitro* mitochondrial function studies, the rat immortalized mesencephalic dopaminergic neuronal cell line ($1RB_3AN_{27}$, or N27) was cultured in RPMI 1640 containing 2 mM $_L$-glutamine, 50 units of penicillin and 50 µg/ml streptomycin with 0-10% FBS in incubators at 37ºC and 5% CO_2 as previously described by our lab (8).

The lentivirus-based CRISPR/Cas9 KO plasmid, pLV-U6gRNA-Ef1aPuroCas9GFP-TFAM, with the TFAM gRNA target sequence directed against the exon 1 sequence (CPR555e5e4099bf84.98), was purchased from Sigma-Aldrich. To make lentivirus, the lenti-CRISPR/Cas9 TFAM KO plasmid and control plasmid were transfected into 293FT cells using the Mission Lentiviral Packaging Mix (SHP001, Sigma-Aldrich) according to manufacturer's instructions. The lentivirus was harvested 48 h post-transfection and titers were measured using the Lenti-X™ p24 Rapid Titer Kit (632200, Clontech, Mountain View, CA). For stable knockdown of TFAM in N27 cells, 6-well plates containing 0.1 x 10^6/well had lentivirus added the following morning to the media at an MOI of 100. After 24 h, fresh media supplemented with puromycin (50 µg/mL) was added to the cells for stable cell selection.

The wild-type microglial cell line, obtained as a kind gift from Dr. Golenbock at the University of Massachusetts, was used for neuroinflammation experiments and cultured in DMEM containing 2 mM $_L$-glutamine, 50 units of penicillin and 50 µg/ml streptomycin with 2% FBS in 6-well plates at a density of 1.5 x 10^6/well. The cell line was immortalized from

wild-type C57 mouse primary microglia by infection with J2 recombinant retrovirus microglia and has been characterized by Halle et al., (37).

Mitochondrial Oxygen Consumption

The Seahorse XF96 analyzer was used to monitor basal oxygen consumption rate (OCR) in N27 dopaminergic neuronal cells at different stages as previously described (8). Briefly, after pre-equilibrating the cartridge containing the mito-stressors oligomycin (1 µg/ml), FCCP (1 µM), and antimycin A (10 µM) in injection ports, a plate with cells pretreated in serum-free media for 3 h with 0, 10 or 30 µM Mito-apo-C_2 and 1 µM rotenone was placed into the Seahorse analyzer. OCR readouts were measured in pmol/min.

Cell Viability, Aconitase, and NOX2 Activity

The CellTiter-Glo Luminescent Cell Viability (used to measure intracellular ATP levels) and aconitase activity assays were performed per manufacturer's instructions using control and TFAM stable knockdown N27 cells or microglia. The CellTiter-Glo Luminescent Cell Viability assays was performed using control or TFAM stable knockdown N27 cells ($0.8x10^4$/well) in a white opaque-walled 96-well plate.

For the aconitase activity assay, $1x10^6$ cells were plated in a T25 flask. After treatments, mitochondrial fractions were collected and lysed per manufacturer's instructions. Readings at 450 nm were obtained using a SpectraMax plate reader and normalized to protein concentrations determined by Bradford assay.

NADPH oxidase activity was determined in differentiated human acute promyelocytic leukemia (*d*HL60) cells, as recently reported (73,75). Hydropropidine probe

(20 µM), in the presence of DNA (0.1 mg/ml), was used to monitor superoxide production, while coumarin boronic acid (100 µM) was used to monitor H_2O_2 formation. HL60 cells were maintained and differentiated with ATRA (1 µM) for five days as described previously (73). The differentiated cells were pretreated with Mito-apo for 30 min in HBSS supplemented with HEPES buffer (25 mM, pH 7.4) and dtpa (0.1 mM), followed by the addition of the probe and PMA (1 µM). Immediately after addition of PMA, the plates were placed in the plate reader and fluorescence intensity monitored over 2 h. The slope of the fluorescence intensity over time was used to measure the probe oxidation rate. The rate of probe oxidation in samples treated with pure DMSO (0.1% vol/vol) was taken as 100%.

Oxidation of mitochondrial boronate probe

Mitochondria-targeted boronates were proposed for site-specific detection of mitochondrial H_2O_2 (10,11). For adjusting the results for differential uptake/availability of the probe in mitochondria, the use of a ratio of the phenolic product to probe level was proposed (10,11). The *o*-MitoPhB$(OH)_2$ probe (74,75) was incubated with microglial cells (50 µM, 1 h) prior to collecting the cells. The samples were prepared and analyzed by LC-MS/MS using the deuterated analogs d_{15}-MitoPhB$(OH)_2$ and d_{15}-MitoPhOH as the internal standards, as described recently (74,75).

MitoTracker and MitoSOX

N27 dopaminergic cells grown on poly-D-lysine-coated glass coverslips in 24-well plates were treated in 2% FBS media for 12 h with 0, 10 or 30 µM Mito-apo-C_2 or 1 µM rotenone, and then washed twice with PBS. CMXRos MitoTracker Red dye (working

concentration of 200 nM) was incubated at 37ºC for 12 min. ImageJ was used to quantify mitochondrial parameters as described previously (8,14). MitoSOX reagent (working concentration of 5 µM) was used to detect mitochondrial oxidant(s) production in live N27 cells per manufacturer's instructions. It should be noted that MitoSOX fluorescence does not allow for identification of the specific type of oxidant(s) detected.

Nitric Oxide and Cytokine Detection

Microglia were co-treated for 12 h with 1 µg/ml LPS and 0, 10, or 30 µM Mito-apo-C_{11}. Supernatant was used to determine extracellular cytokine levels using the Luminex bead-based immunoassay with recombinant standards for IL-6, IL-12, TNF-α, and IL-1β. Nitric oxide production was measured as media nitrite levels using Griess reagent and sodium nitrite standard curve as previously described (35).

Animals and Treatments

MitoPark mice were a kind gift of Dr. Nils-Goran Larson at the Max Planck Institute for Biology and Aging in Cologne, Germany. All mice for this study were bred and housed under standard conditions (*ad libitum food,* a 12-h light cycle, constant temperature and humidity) at Iowa State University (ISU) as approved by the Institutional Animal Care and Use Committee. MitoPark and age-matched control mice were oral gavaged with either 10 mg/kg Mito-apo-C_{11} dissolved in 10% ethanol in PBS or an equivolume vehicle solution thrice weekly from age 13 to 24 wks. Mice were subjected to behavioral testing, and neurochemical and histological measurements were performed at age 24 wks.

Behavior

Open-field and RotaRod experiments were performed as previously described (32). Briefly, open-field measurements of spontaneous locomotor activity were accomplished using VersaMax (monitor RXYZCM-16, analyzer VMAUSB, AccuScan, Columbus, OH) during a 10-min acquisition period following a 2-min accustomization. Coordination was assessed by RotaRod (AccuScan) performance at 20 rpm for five trials not exceeding 20 min.

HPLC Analysis of Neurotransmitters

Striatal lysates were prepared in an antioxidant buffer containing 0.2 M perchloric acid, 0.1% $Na_2S_2O_5$, 0.05% NaEDTA, and isoproterenol as an internal standard as previously described (33). Samples were sonicated for 2 min, centrifuged for 10 min, and filtered through a 0.2 µm filter prior to running. Samples were placed into an autosampler (WPS-3000TSL, Thermo Scientific) at 4ºC and separated isocratically by a reverse-phase C18 column at 0.6 ml/min (pump, Ultimate 3000 ISO-3100SD). Electrochemical detection (Coularray 5600A) was performed using potentials of 350, 0, -150, and 220 mV (microdialysis cell 5014B, guard cell 5020) to detect dopamine, DOPAC, and HVA. Chromatograph integration and analyses were performed using Chromeleon (7.1.3) and ESA Coularray (3.10) Software.

Western Blotting

Western blot lysates from the striatum and SN were prepared in RIPA buffer with protease and phosphatase inhibitors (Pierce Biotechnology) and ran on a 10-15% SDS-PAGE as previously described (33). Following SDS-PAGE electrophoresis, proteins were

transferred to a nitrocellulose membrane (Bio-Rad). After blocking for 1 h in Licor blocking buffer or milk, primary antibodies including 4-HNE (1:2000), TH (1:1500), iNOS (1:500), IBA1 (1:1200), and β-actin (1:7000) were incubated at 4ºC overnight. Secondary antibodies (Alexa Fluor 680 and Rockland IR800) were incubated at RT for 1h and images were captured via Licor Odyssey imager. Densitometric analysis was done using ImageJ software.

Immunohistochemistry (IHC) and Confocal Imaging

Perfusion, sectioning, and IHC were performed as previously described by Ghosh et al. (33). Animals were transcardially perfused with PBS and 4% PFA. The next day, brains were cryoprotected with 30% sucrose and brain blocks were made in OCT. Cryosolution containing ethylene glycol and sucrose was used to store 30-µm sections until use. Following washing, antigen retrieval was achieved by keeping sections at 80ºC for 30 min in sodium citrate. Sections were incubated with primary antibodies overnight at 4ºC. Appropriate secondary antibodies (Alexa Fluor 488 and 555) were incubated with sections for 1.5 h, followed by 7 min with Hoechst stain. Dehydrated slides were mounted using DPX.

Images (20-30x) were captured with an inverted microscope attached to a SPOT digital camera. For confocal imaging, the ISU Microscopy Facility's Leica DMIRE2 confocal microscope was used to acquire 63x z-stack images, which were converted to maximum projection images using Imaris software.

DAB Immunostaining and Cell Counting

For DAB staining, IHC was performed on 30-µm SN sections. A biotinylated secondary antibody followed by incubation with an avidin peroxidase solution (Vector labs)

was used to yield a brown stain after incubation with DAB solution. Stereological counting of TH^+ neurons was done on every 6^{th} section at 40x using Stereo Investigator software (MicroBrightField) with an optical fractionator. Images (20x) for $IBA1^+$ microglia analysis were acquired with Spot software. ImageJ software was used to count cells, adjust threshold, convert images to binary, skeletonize, and analyze skeleton (49).

Electron Paramagnetic Resonance (EPR)

EPR spectra were recorded on a Bruker EleXsys E600 spectrometer equipped with a Super-X microwave bridge with integrated microwave counter, an ER4112SHQ resonant cavity operating at 9.38 GHz, and an Oxford Instruments ESR900 helium flow cryostat with an ITC503 temperature controller. Spectra were recorded at 12 K with 5 mW microwave power and 10 G magnetic field modulation at 100 kHz; this modulation amplitude determined the spectral resolution. Scans of 4096 points, an 8000 G field envelope and 3-min duration were averaged over 60-180 min to provide the final spectrum. A background spectrum collected on frozen water was subtracted from experimental spectra. Contributions of each of the paramagnetic centers of the mitochondrial proteins to the experimental spectra were estimated by fitting a library of computed spectra corresponding to the mitochondrial respiratory chain centers and the [3Fe4S] cluster of aconitase, using a Levenberg-Marquardt algorithm to minimize χ^2 (IGOR Pro v. 6.32A, Wavemetrics, Lake Oswego, OR). The contributions of each component were constrained to ≥0. Computed spectra of the individual components were calculated, using spin Hamiltonian parameters from the literature (23,26,36,40,50,58,72), with XSophe (Bruker Biospin; (38)).

Detection of Mito-apo-C11 in Brain and Blood

Mice were administered 10 mg/kg Mito-apo-C11 by oral gavage and then sacrificed at 30 min, 3, 6, 12, 24 or 48 h (n=3/time point). Blood was collected by cardiac puncture into heparinized tubes for plasma, and various brain regions were dissected out and stored at -80ºC. Tissue homogenates were then made using antioxidant buffer, as described above for neurotransmitter extraction. The standards prepared and used for quantification were 0.1, 0.3, 1.0, 10, and 30 µg. Samples were centrifuged and 0.2 µm-filtered before analysis on an Agilent Technologies 1200 Series HPLC system using a 20-µl injection volume. Separation was performed with a Kinetex C18 column, operated at a flow rate of 1.5 ml/min and a temperature of 40°C, using a gradient elution (solvent A= 90% water, 10% acetonitrile, solvent B= 100% acetonitrile) over 10 min. Mito-apo-C11 was quantified by UV absorbance (collected at wavelengths of 262 nm and 292 nm) with an average retention time of 6.35 min. Standards were fit to a linear regression and data were analyzed using GraphPad Prism 4.0.

Statistical Analysis

Data were analyzed by one-way ANOVA with Bonferroni post-test using Prism 4.0 software. Differences with p-values ≤ 0.05 were considered significant.

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Author Contributions

A.G.K., M.L, and A.G. conceived and designed the experiments. M.L., A.G., A.C., J. Z., B.S., B.B., T.B., and S.S. performed the experiments. M.L., A.G., J. Z., B.B., T.B., and B.N. analyzed the data. M.A., J.L., S.G., D.K, contributed reagents, cell lines, and mouse strains. M.L., H.J, and V.A. wrote the paper. A.G.K., A.K. and B.K. led the investigation, conceived the project, and wrote the manuscript. All authors reviewed and edited the manuscript.

Author Disclosure Statement

A.G.K. and B.K. hold a patent entitled “Neuroprotective Compounds and Their Use” for the Mito-apocynin treatment of Parkinson’s disease. A.G.K. and V.A. are shareholders of PK Biosciences. The other authors have no conflicts of interest.

Abbreviations Used

4-HNE= 4-hydroxynonenal

6-OHDA= 6-hydroxydopamine

ALP= alkaline phosphatase

ATRA= all-trans retinoic acid

CNS= central nervous system

CRISPR= clustered regularly interspaced short palindromic repeats

DOPAC= 3,4-dihydroxyphenylacetic acid

EPR= electron paramagnetic resonance

FBS= fetal bovine serum

HVA= homovanillic acid

IBA1= ionized calcium-binding adapter molecule 1

iNOS= inducible nitric oxide synthase

HPLC= high performance liquid chromatography

KD= knockdown

L-DOPA= L-3,4-dihydroxyphenylalanine

LRRK2= leucine-rich repeat kinase 2

Mito-apo= Mito-apocynin

MTA= mitochondria-targeted antioxidants

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

NOX=NADPH oxidase

OCR= oxygen consumption rate

PINK1= PTEN-induced putative kinase 1

PMA= phorbol myristate acetate

PD= Parkinson's disease

ROS= reactive oxygen species

SN= substantia nigra

TFAM= mitochondrial transcription factor A

TH= tyrosine hydroxylase

TNFα= Tumor necrosis factor α

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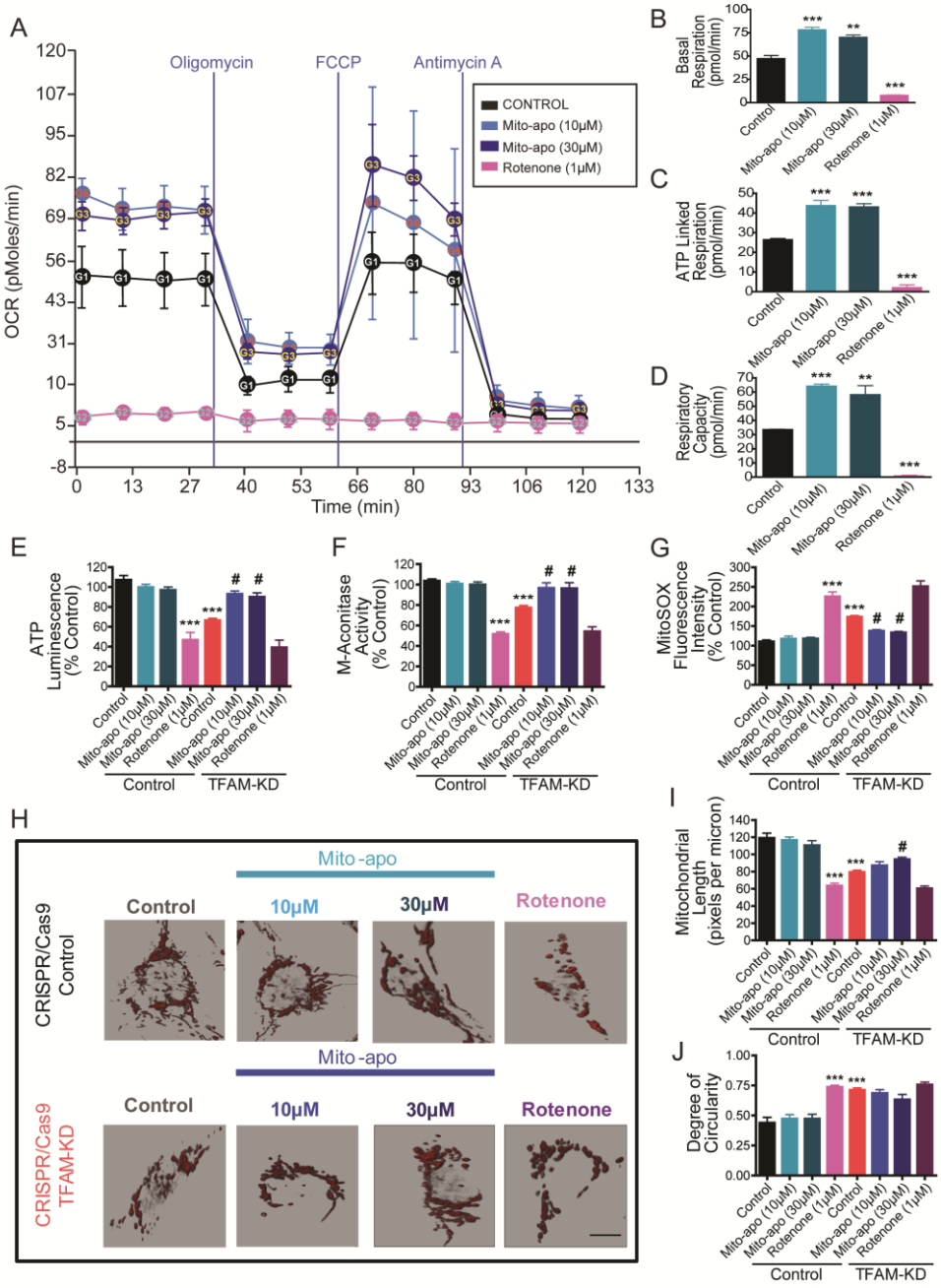

Figure 1. Bioenergetics and *in vitro* characterization of Mito-apo. *A-D*, N27 cells given 10-30 μM Mito-apo for 3 h were measured using a Seahorse XF96 analyzer. Basal respiration rate prior to injection *(A, B).* After adding oligomycin, ATP-linked respiration *(A,*

C). Respiratory capacity measured as OCR after FCCP injection *(A, D). E-G,* Mito-apo replenished ATP levels (*E*), upregulated m-aconitase activity (F) and decreased oxidant production in TFAM knockdown cells (TFAM-KD). TFAM-KD and control cells were incubated with 10-30 µM Mito-apo for 3 (*E-F*) or 12 h (*G*). Raw luminescence units (RLU) of ATP produced (*E*). Aconitase activity *(F)* and mitochondrial ROS production *(G)* as determined by aconitase and MitoSOX assays. *H-J,* TFAM-KD and control cells were incubated with 10-30 µM Mito-apo for 12 h, and mitochondria were stained by MitoTracker (scale bar=10 µm). Analysis of mitochondrial length *(I)* and degree of circularity *(J).* Rotenone (1 µM) was used as a positive control in all experiments. Graphical results represented as the mean±SEM (n=6 wells/group). *, $p<0.05$ versus control; **, $p<0.01$ versus control; ***, $p<0.001$ versus control; #, $p<0.05$ versus vehicle-treated TFAM-KD cells. See also Figures S1 and S6.

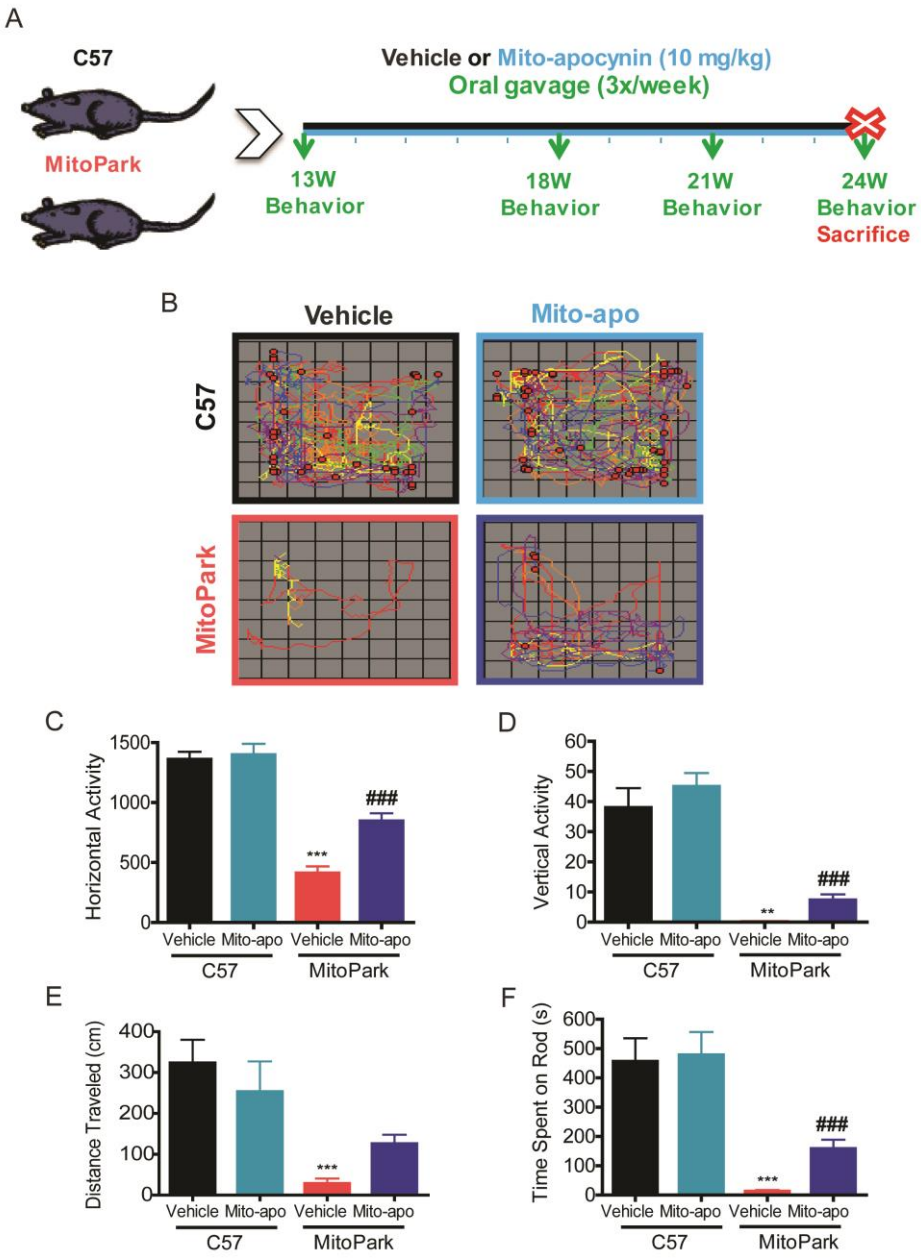

Figure 2. Progressive motor deficits in MitoPark mice are improved with Mito-apo. *A*, Treatment schedule showing C57 and MitoPark (MP) mice orally administered vehicle or Mito-apo (10 mg/kg). *B*, VersaPlot showing horizontal activity and rearing activity (red dots) during a 10-min open-field test. Horizontal *(C)* and vertical *(D)* activities and distance

traveled *(E)* during open-field test. *F*, Time spent on RotaRod. Graphical results represented as the mean±SEM (n=9-13 mice/group). ###, $p<0.001$ versus vehicle-treated MP. See also Figure S5.

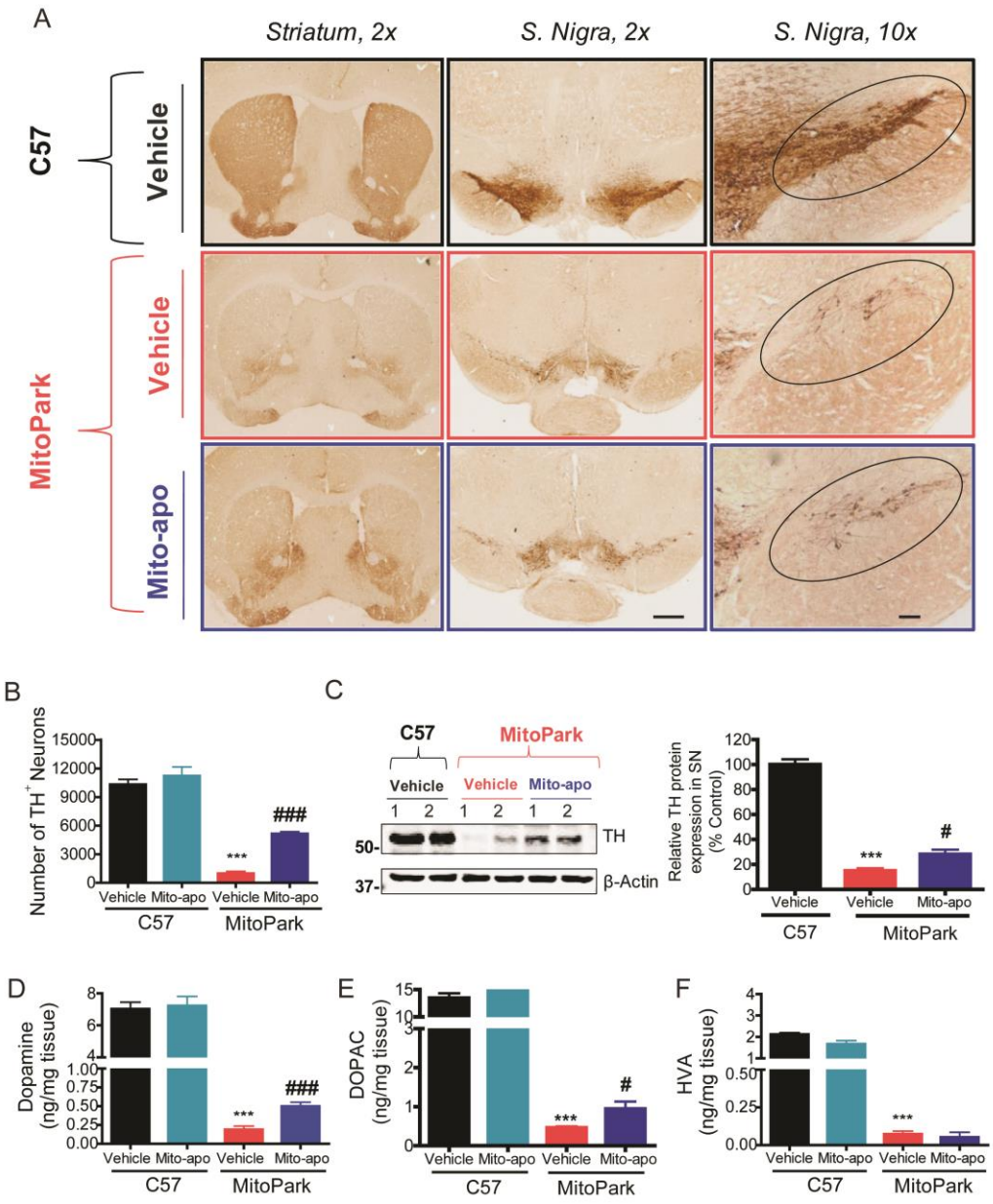

Figure 3. Neurodegeneration and dopamine depletion in MitoPark mice is attenuated by Mito-apo. DAB immunostaining *(A)* in striatum and SN (scale bars=1 mm and 100 µm) of vehicle-treated C57 (top), vehicle-treated MP (middle), and Mito-apo-treated MP mice (bottom). *B*, Stereological cell counts of tyrosine hydroxylase $(TH)^{+}$ neurons in the SN of

n=3/group. *C,* Representative Western blot of two animals/group and densitometric analysis of TH protein levels normalized to β-actin in the SN (n=6/group). HPLC with electrochemical detection was used for determination of the neurotransmitters dopamine *(D),* DOPAC *(E),* and HVA *(F).* Graphical results represented as the mean±SEM (n=6-8 mice/group). #, $p<0.05$, ##, $p<0.01$, or ###, $p<0.001$ versus vehicle-treated MP.

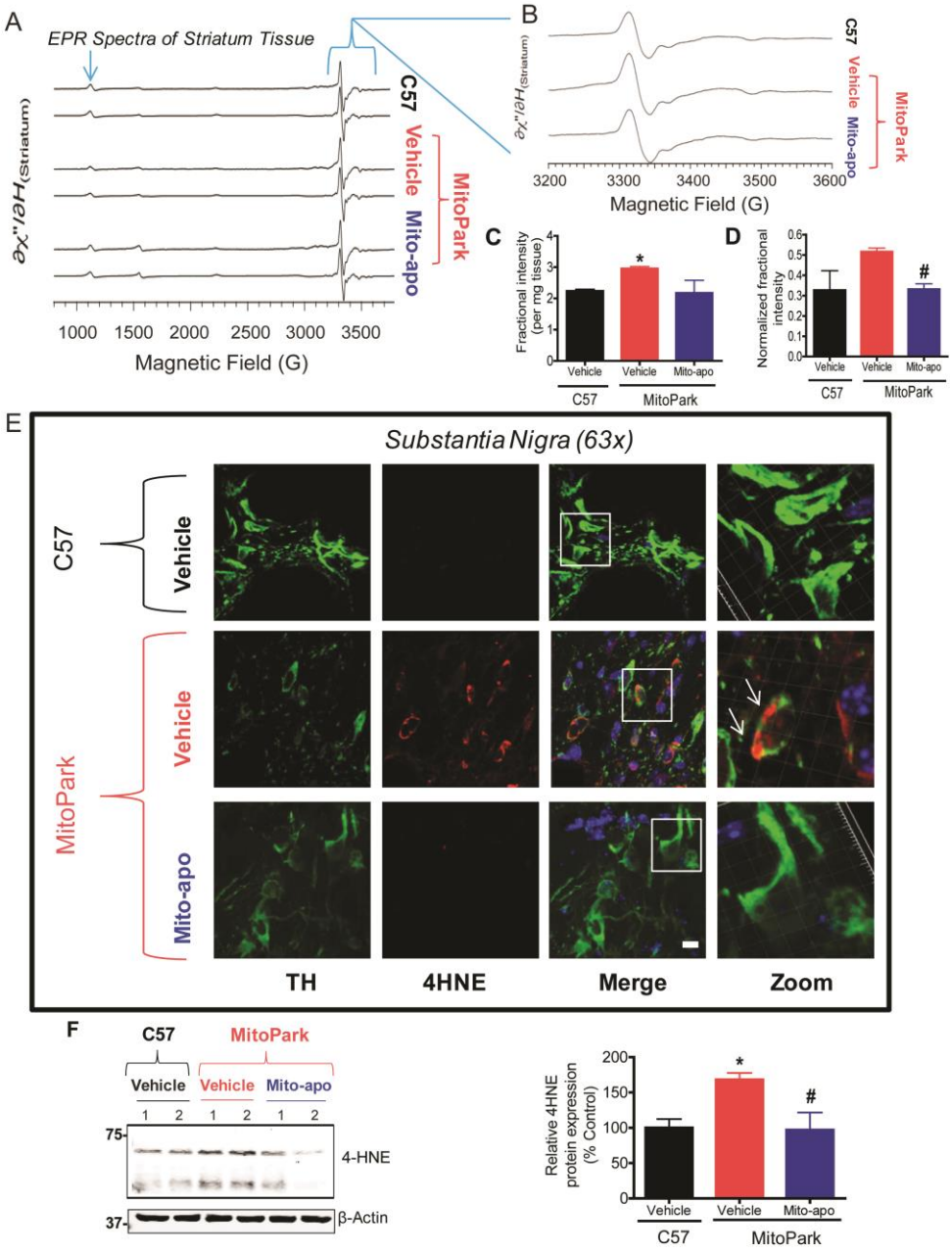

Figure 4. Mitochondrial aconitase inactivation and oxidative stress are attenuated in Mito-apo-treated MitoPark mice. *A*, The *g* ~ 2 region (blue bracket) and HS heme (arrow, internal control) of the experimental EPR spectra from the pooled striatum (each spectrum represents 3-5 combined animal tissues) from vehicle-treated C57 (top), vehicle-treated

MitoPark (MP) (middle), and MP mice treated with 10 mg/kg Mito-apo from 13-24 wks of age (bottom). The spectra are primarily due to the oxidized [3Fe4S] cluster of aconitase (~3330 G), and the reduced N2 (3370 G), N4 (3485 G), and N3 (3540 G) complex I iron-sulfur complexes, although other components underlie these species, and fits to all components were used to estimate the relative contributions to the spectra (fits are presented under each of the three experimental traces, respectively, in Panel A). *B,* Expansion of the *g* ~ 2 region. Fractional intensities from EPR spectra in the *g* ~ 2 region normalized to combined tissue weight *(C*), and internally normalized to the HS-heme group *(D). E,* 24-wk mouse tissues of vehicle-treated C57 (top), vehicle-treated MP (middle), and Mito-apo-treated MP mice (bottom) were double immunostained for TH and 4-HNE (scale bar=10µm). Merged MP image shows co-localization of TH and 4-HNE in DAergic neurons. *F,* Representative Western blot and densitometric analysis of 4-HNE protein in the SN (n=6/group). *, $p<0.05$ versus C57; #, $p<0.05$ versus vehicle-treated MP. See also Figure S2.

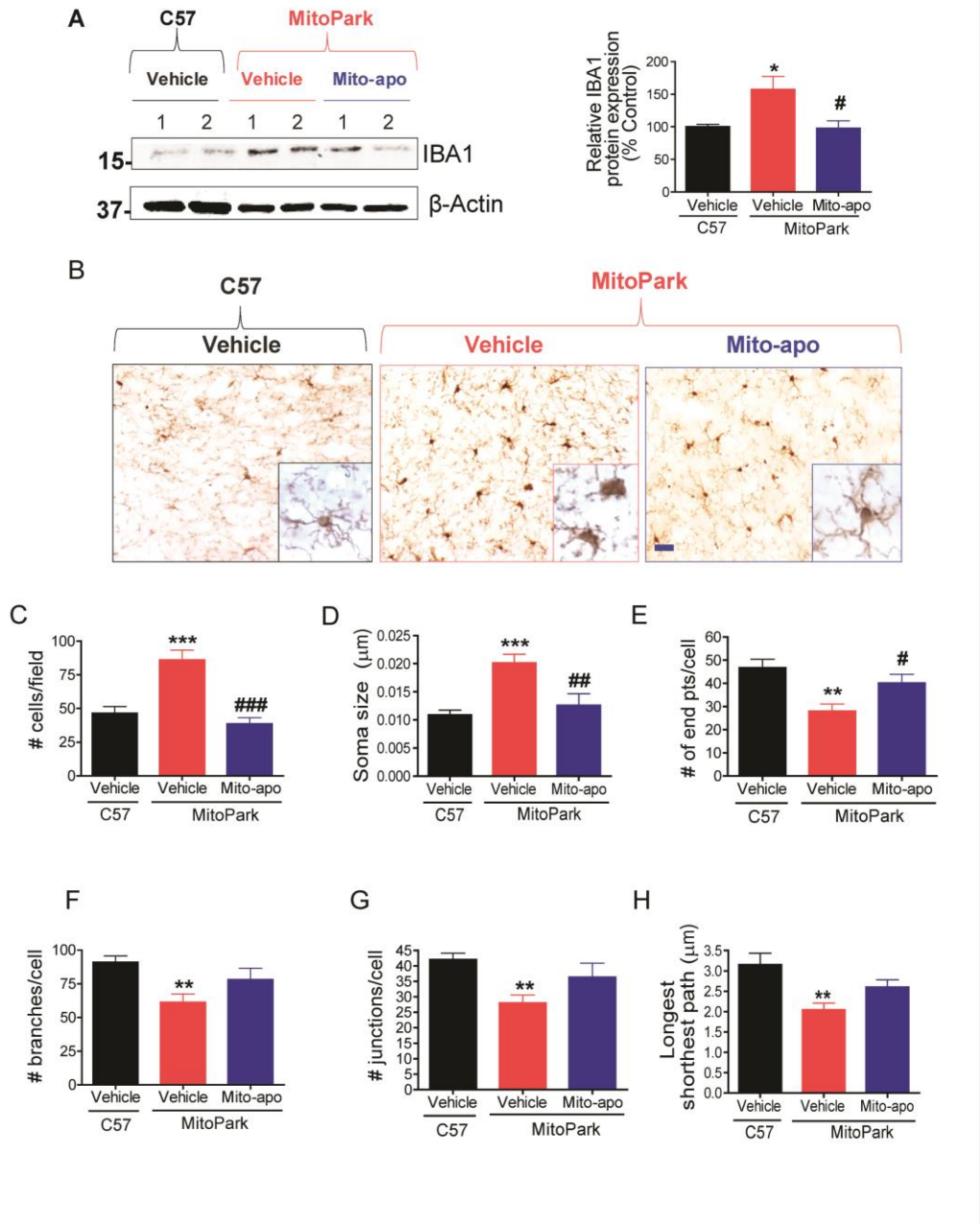

Figure 5. Mito-apo inhibits microglia activation in the substantia nigra of MitoPark mice. *A*, Western blot and densitometric analysis of IBA1 protein in the SN (n=6/group). *B*, IBA1 DAB immunostained sections from 24-wk mouse SN of vehicle-treated C57 (left), vehicle-treated MitoPark (MP) (middle), and Mito-apo-treated MP mice (right) showing

microglia morphology (scale bar=200μm)ImageJ analysis of microglia for cells per field *(C*), soma size *(D*), number of end points per cell *(E*), number of branches per cell *(F*), number of junctions per cell (*G*), and longest shortest path (*H)*. Graphical results represented as the mean±SEM (n=5-7 mice/group). *, $p<0.05$ versus C57 control; #, $p<0.05$ versus vehicle-treated MP; ##, $p<0.01$ versus vehicle-treated MP; ###, $p<0.001$ versus vehicle-treated MP. See also Figure S7.

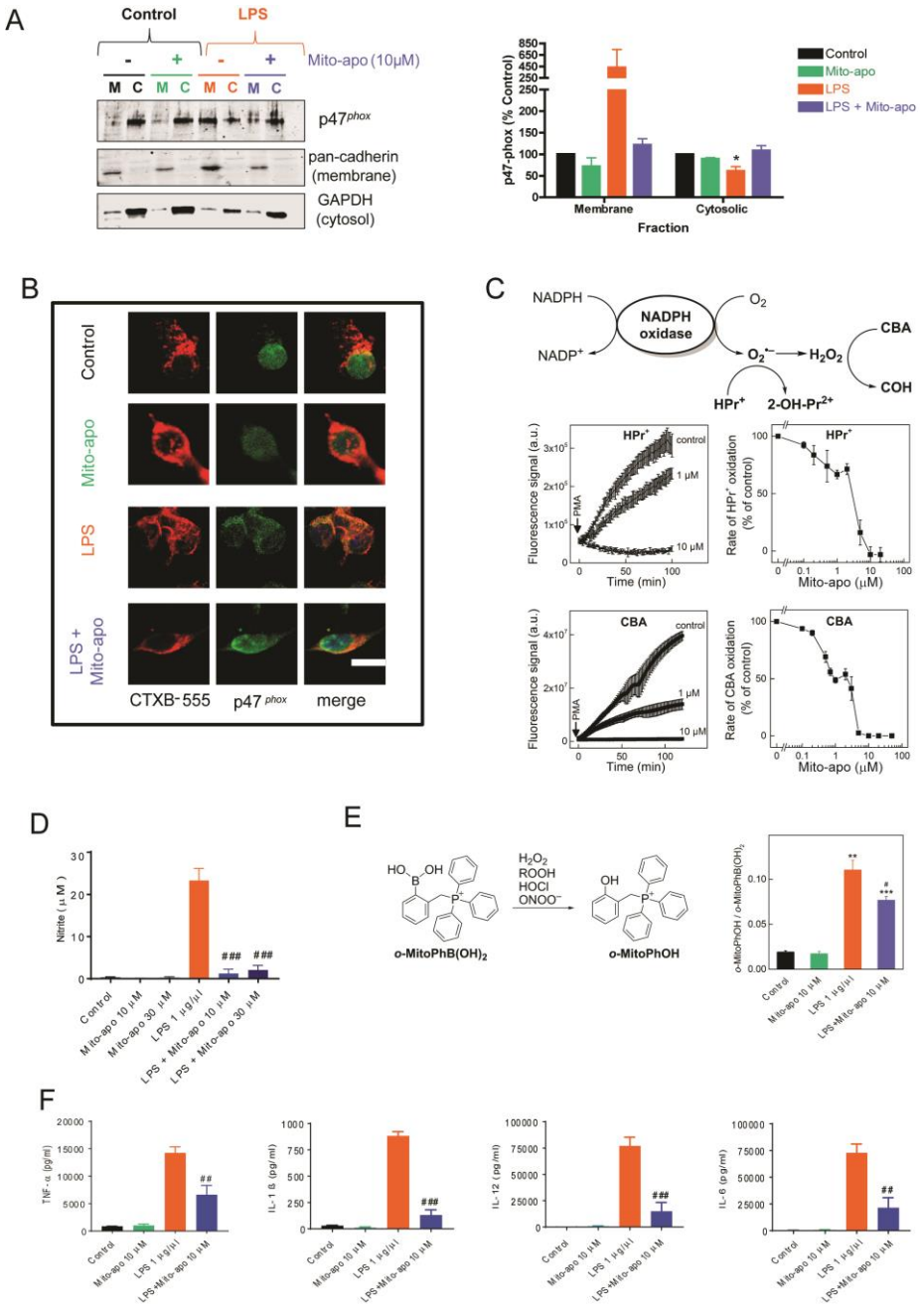

Figure 6. Mito-apo decreases NOX2 and iNOS activity in microglia. *A*, Western blot and accompanying densitometric analysis of membrane and cytosolic fractions from control and LPS-stimulated microglia treated with or without 10 μM Mito-apo. B, Immunocytochemistry revealing increased membrane co-localization of $p47^{phox}$ upon LPS-stimulation that is

reduced with Mito-apo treatment. CTXB= cholera toxin subunit B with 555 fluorophore. *C*, ATRA-differentiated HL60 cells were pretreated with Mito-apo for 30 min in HBSS supplemented with HEPES buffer (25 mM, pH 7.4) and dtpa (0.1 mM), followed by the addition of the probe and PMA (1 μM). The slope of the fluorescence intensity monitored over 2 h was used to measure the probe oxidation rate. *D*, Nitric oxide production from microglia as estimated in supernatant by Griess assay of nitrite levels. *E*, LPS-induced oxidation of *o*-MitoPhB$(OH)_2$ to *o*-MitoPhOH in microglia was inhibited by Mito-apo. *F*, Luminex multiplex assay showing levels of TNF-α, IL-1β, IL-6 and IL-12 in supernatant from LPS-stimulated microglia (n=4-6/group). Graphical results represented as the mean±SEM (n=3/group). *, $p<0.05$ versus C57 control. See also Figure S6.

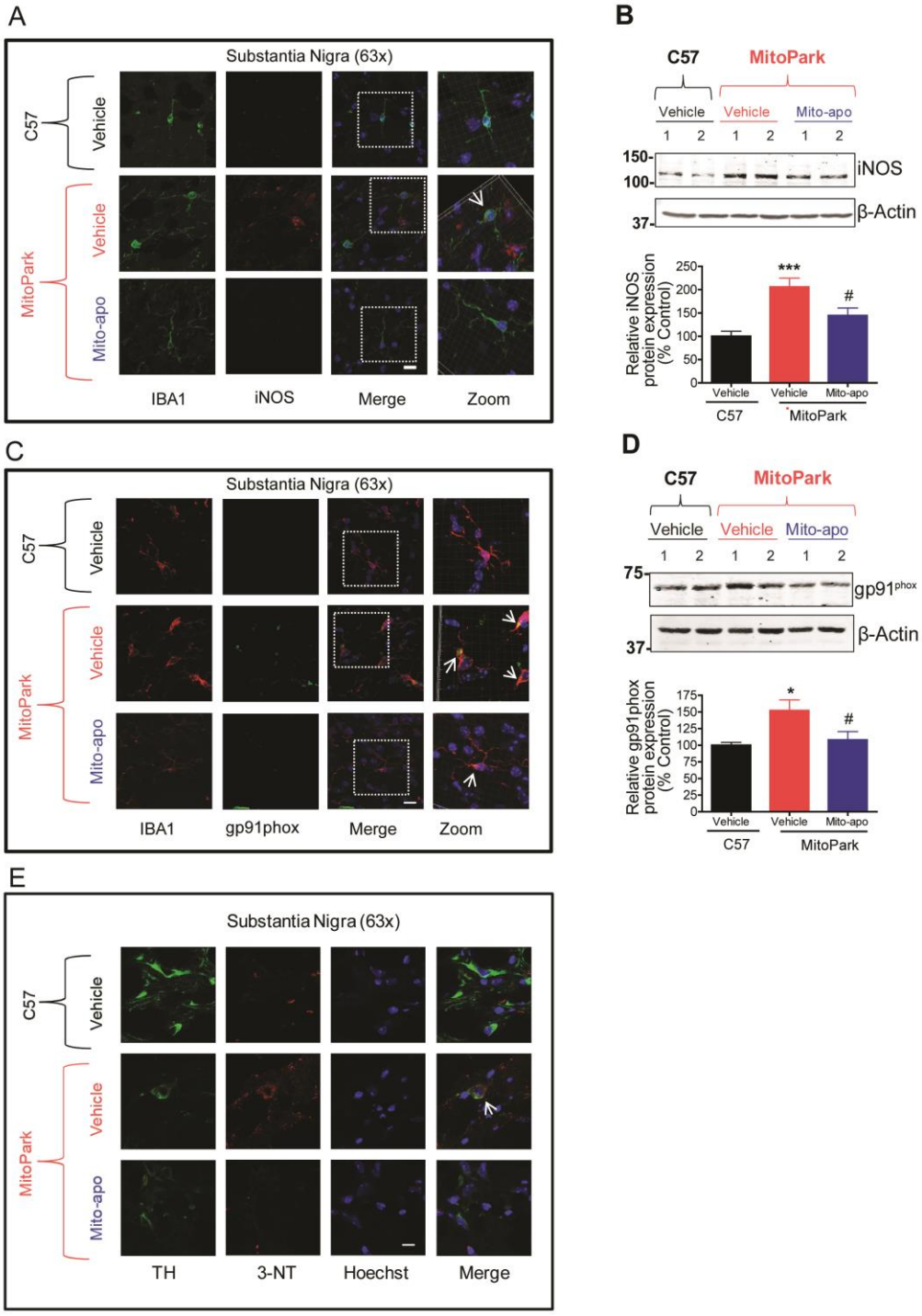

Figure 7. Mito-apo inhibits iNOS and NOX2 expression in the substantia nigra of MitoPark mice. Determination of NOX2 and iNOS activity in the SN. *A*, 24-wk mouse

tissues of vehicle-treated C57 (top), vehicle-treated MP (middle), and Mito-apo-treated MP mice (bottom) stained for IBA1 and iNOS (scale bar=10 μm). Merged MP image shows co-localization (arrow) of IBA1 and iNOS in SN tissues. *B*, Representative Western blot and densitometric analysis of iNOS protein in the SN (n=6/group). *C*, IHC for IBA1 and $gp91^{phox}$ of vehicle-treated C57 (top), vehicle-treated MP (middle), and Mito-apo-treated MP mice (bottom). Merged MP image shows co-localization (arrows) of IBA1 and $gp91^{phox}$ in SN tissues. *D*, Representative Western blot and densitometric analysis of $gp91^{phox}$ protein in the SN (n=6/group). *E*, IHC for TH and 3-nitrotyrosine of vehicle-treated C57 (top), vehicle-treated MP (middle), and Mito-apo-treated MP mice (bottom). Graphical results represented as the mean±SEM. #, $p<0.05$ versus vehicle-treated MP; ##, $p<0.01$ versus vehicle-treated MP; ###, $p<0.001$ versus vehicle-treated MP.

Table 1. Clinical pathology results for mice (n=3) 3h post-oral gavage with 10 mg/kg Mito-apo-C_{11} using the Abaxis system.

			3-hr Control				3-hr Mito-apo-C_{11}			
Parameter	**Units**	**p-value**	**Mean**	**Min**	**Max**	**SD**	**Mean**	**Min**	**Max**	**SD**
Albumin	**g/dl**	0.31	3.23	2.40	3.90	0.62	3.77	3.50	4.00	0.21
Alk phos	**IU/L**	1.00	70.67	56.00	88.00	13.20	70.67	63.00	81.00	7.59
ALT	**IU/L**	0.41	65.67	38.00	84.00	19.91	94.00	54.00	146.00	38.51
Amylase	**U/L**	0.38	1061.33	927.00	1316.00	180.17	1699.00	1050.00	2977.00	903.72
Total bilirubin	**U/L**	0.37	0.30	0.30	0.30	0.00	0.33	0.30	0.40	0.05
BUN	**mg/dl**	0.83	15.33	12.00	21.00	4.03	14.67	13.00	16.00	1.25
Calcium	**mg/dl**	0.50	11.73	11.50	12.20	0.33	11.50	11.20	11.90	0.29
Phosphorous	**mg/dl**	0.94	10.20	8.50	12.20	1.53	10.30	9.50	11.80	1.06
Creatinine	**mg/dl**	0.37	0.27	<0.20	0.40	0.09	0.20	<0.20	<0.20	0.00
Glucose	**mg/dl**	0.05	292.33	274.00	317.00	18.12	336.67	320.00	352.00	13.10
Sodium	**mEq/L**	0.84	159.00	156.00	161.00	2.16	159.33	159.00	160.00	0.47
Potassium	**mEq/L**	1.00	8.50	>8.50	>8.50	0.00	8.50	>8.50	>8.50	0.00
Total Protein	**g/dl**	0.81	5.50	5.40	5.60	0.08	5.53	5.30	5.70	0.17

Table 2. Clinical pathology results for mice (n=3) 48 h post-oral gavage with 10 mg/kg Mito-apo-C_{11}.

			48-hr Control				48-hr Mito-apo-C_{11}			
Parameter	**Units**	**p-value**	**Mean**	**Min**	**Max**	**SD**	**Mean**	**Min**	**Max**	**SD**
Albumin	**g/dl**	0.23	4.07	3.70	4.40	0.29	4.37	4.30	4.50	0.09
Alk phos	**IU/L**	0.02	134.00	119.00	159.00	17.80	82.00	72.00	91.00	7.79
ALT	**IU/L**	0.20	60.33	45.00	77.00	13.10	90.33	58.00	118.00	24.72
Amylase	**U/L**	0.05	858.33	816.00	935.00	54.31	1024.00	962.00	1118.00	67.59
Total bilirubin	**U/L**	0.52	0.37	0.30	0.40	0.05	0.33	0.30	0.40	0.05
BUN	**mg/dl**	0.15	14.67	13.00	18.00	2.36	17.67	17.00	18.00	0.47
Calcium	**mg/dl**	0.02	10.73	10.50	11.00	0.21	11.40	11.20	11.60	0.16
Phosphorous	**mg/dl**	0.01	8.30	8.10	8.50	0.16	10.03	9.40	10.40	0.45
Creatinine	**mg/dl**	1.00	0.20	0.20	0.20	0.00	0.20	0.20	0.20	0.00
Glucose	**mg/dl**	0.06	227.67	196.00	250.00	23.01	271.67	261.00	282.00	8.58
Sodium	**mEq/L**	0.14	159.00	156.00	161.00	2.16	162.00	161.00	163.00	0.82
Potassium	**mEq/L**	0.37	8.40	8.20	8.50	0.14	8.50	8.50	8.50	0.00
Total Protein	**g/dl**	0.12	5.17	4.90	5.50	0.25	5.53	5.40	5.60	0.09

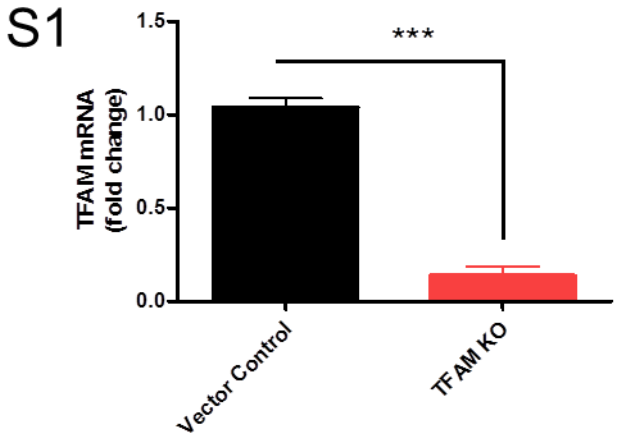

Figure S1. Loss of TFAM in CRISPR/Cas9-dependent knockout N27 cells. Related to Figure 1. Quantitative qPCR analysis showing more than 80% reduction in TFAM mRNA versus control lentivirus-infected N27 cells. ***, $p<0.001$.

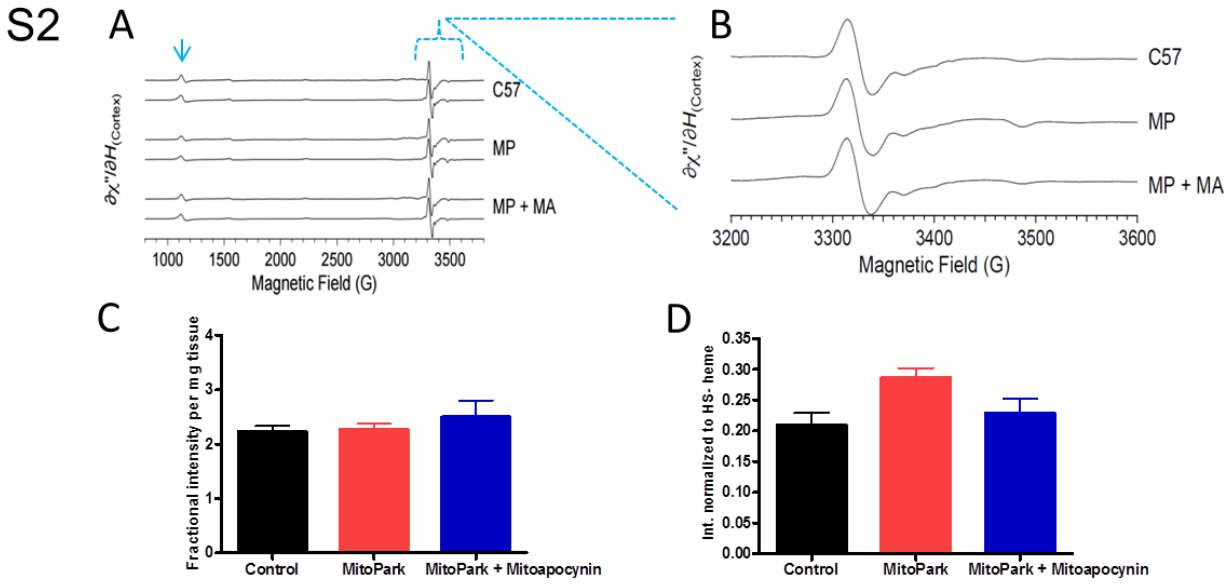

Figure S2. Mitochondrial aconitase activity in Cortex. Related to Figure 4. *A*, The *g′* ~ 2 region (bracket) and HS heme (arrow, internal control) of the experimental EPR spectra from the cortex of vehicle-treated C57 (top), vehicle-treated MitoPark (MP, middle), and Mito-apo-treated MitoPark (MP+MA, bottom) mice are presented. The spectra are primarily due to the oxidized [3Fe4S] cluster of aconitase (at around 3330 G), and the reduced N2 (3370 G), N4 (3485 G), and N3 (3540 G) Complex I iron-sulfur complexes, although other components underlie these species, and fits (not shown) to all components were used to estimate the relative contributions to the spectra. *B*, Expansion of the *g′* ~ 2 region. Quantification of fractional intensities from EPR spectra in the *g′* ~ 2 region normalized to combined tissue weight *(C)*, and internally normalized to the HS-heme group *(D)*.

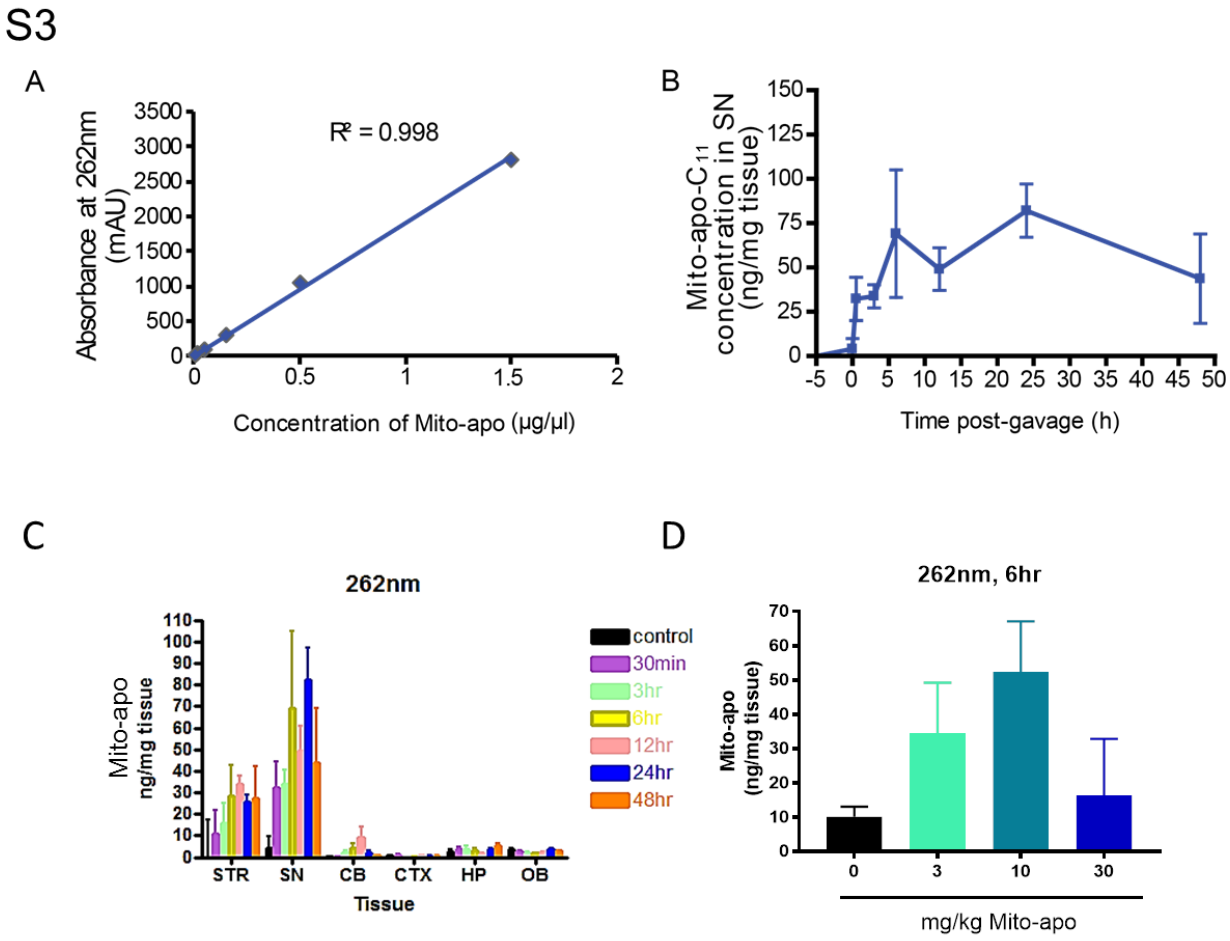

Figure S3. Pharmacokinetic profile of Mito-apo. A, Standard curve of Mito-apo detected by HPLC-UV. Mice were orally administered Mito-apo (10 mg/kg), sacrificed, and tissues were harvested at 30 min, 3, 6, 12, 24 and 48 h. *B*, Quantification of Mito-apo in the SN (n=3/group) at time points following a single gavage of 10 mg/kg Mito-apo. *C*, Quantification of Mito-apo in brain regions of mice (n=3/group) at various time points following a single gavage of 10 mg/kg Mito-apo. *D*, Quantification of Mito-apo detected in the striatum of mice (n=3/group) six hours after oral administration of 0, 3, 10, or 30 mg/kg Mito-apo. See also Tables1 and 2.

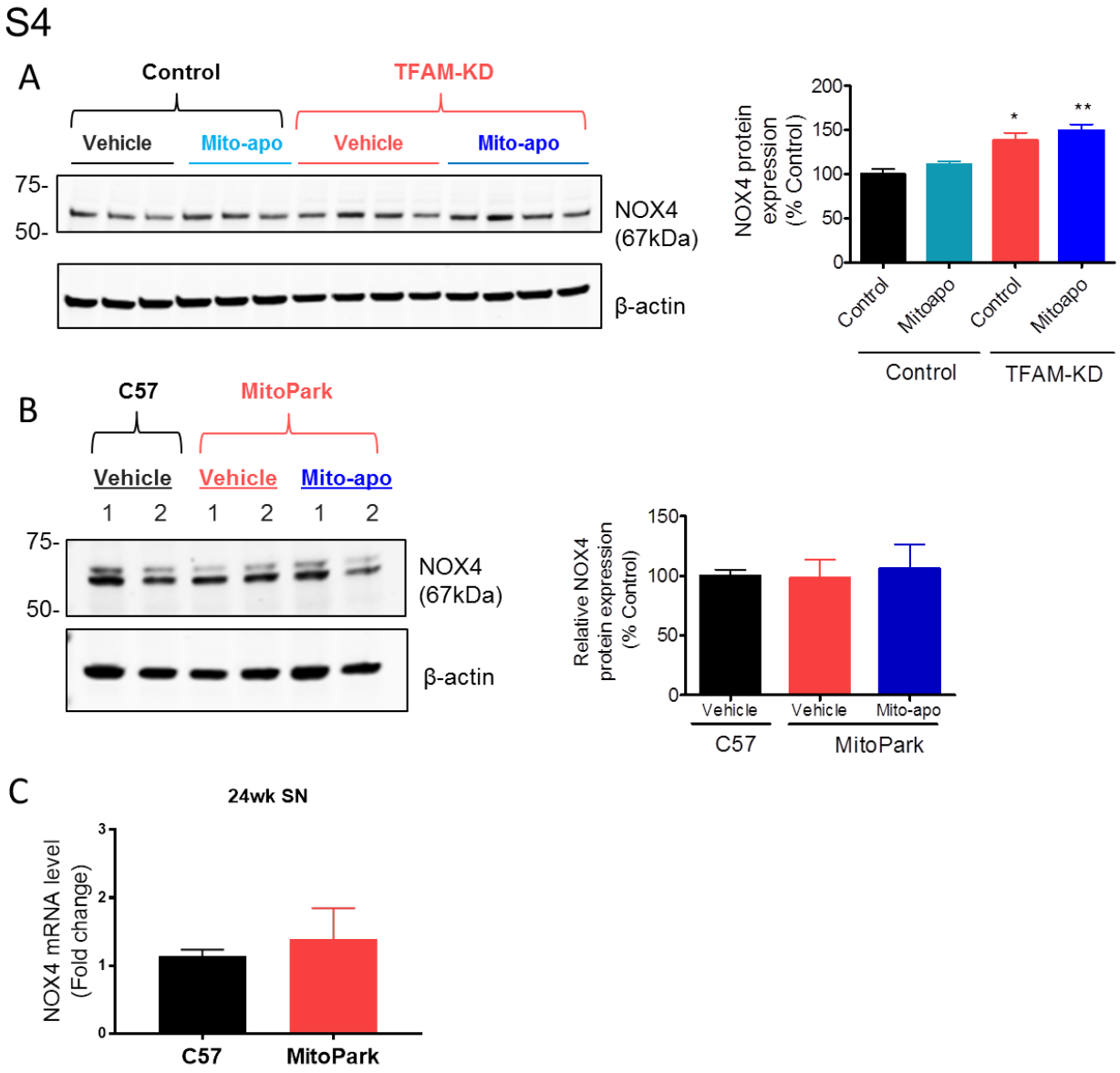

Figure S4. NOX4 is not a primary target of Mito-apocynin. Related to Figure 6. *A*, Western blot and densitometric analysis of NOX4 in Control or TFAM-KD N27 cells. * , $p<0.05$ and **, $p<0.01$ versus vehicle-treated Control N27 cells. *B*, Representative Western blot and densitometric analysis (n=6/group) for NOX4 protein expression in Control and MitoPark mice. *C*, mRNA level of NOX4 in Control (C57) and MitoPark mice (n=3/group).

S5

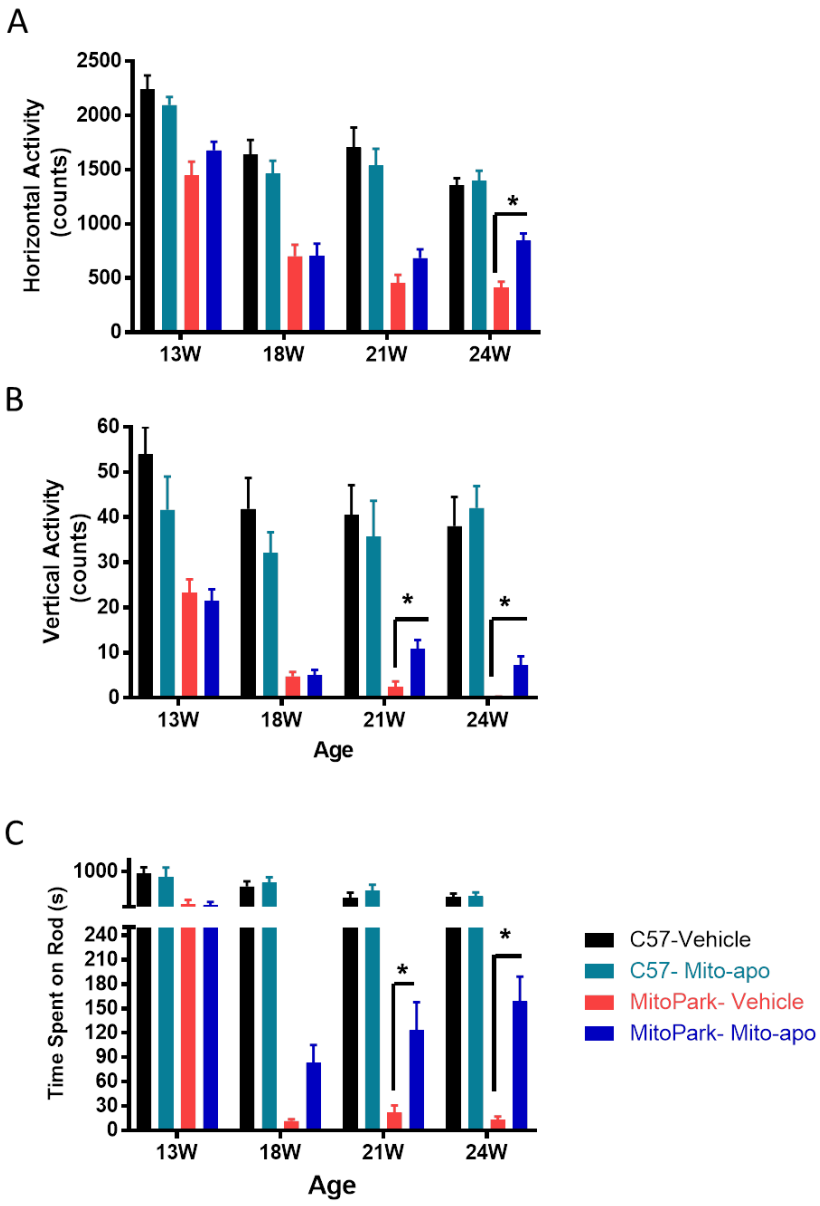

Figure S5. Motor deficits in MitoPark mice at ages 13, 18, and 21 weeks. Related to Figure 2. Horizontal (A) and vertical (B) activity levels during open-field test. C, Time spent on RotaRod. Graphical results represented as the mean±SEM (n=9-13 mice/group). * , $p<0.001$ versus vehicle-treated MitoPark mouse.

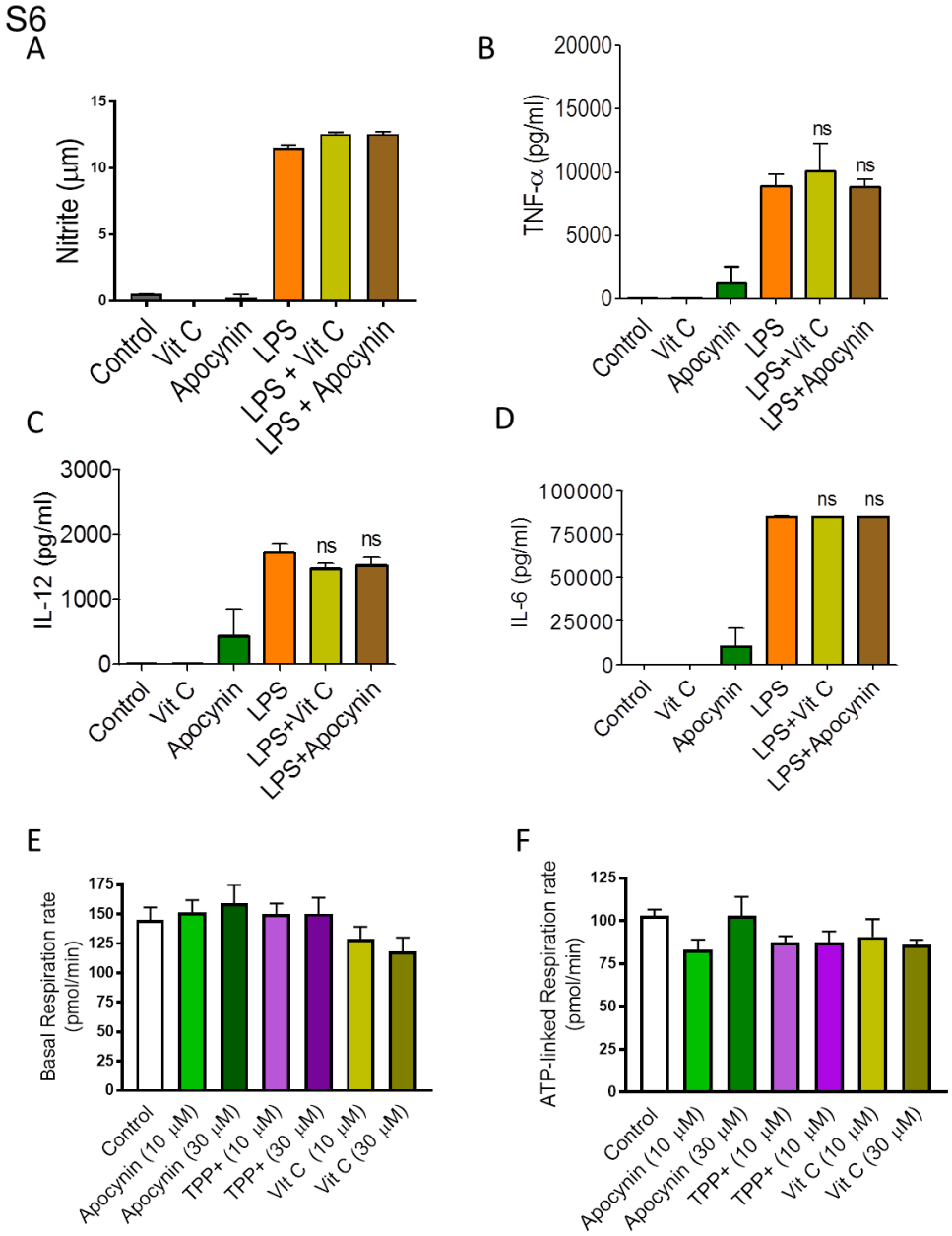

Figure S6. Antioxidant and parent compound controls. Related to Figure 6. Nitric oxide production as determined by Griess assay (*A*) and cytokine levels (*B-D*) in LPS-stimulated microglia co-treated with 10 µM Vitamin C or apocynin. OCR values for basal (*E)* and ATP-linked (*F*) respiration in pmol/min from N27 neuronal cells treated with 10-30 µM Vitamin C, TPP+, or apocynin. ns, not significant.

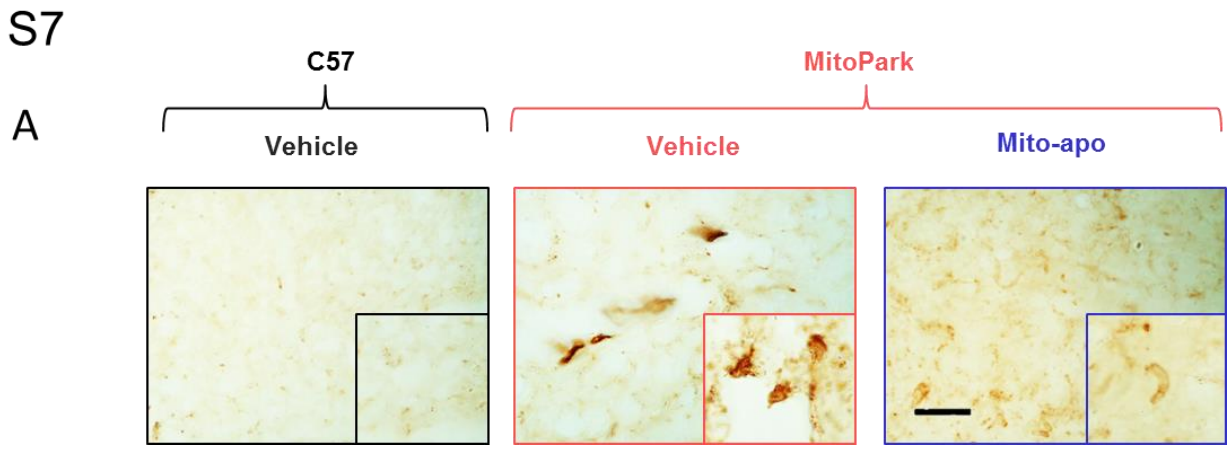

Figure S7. CD68 immunoreactivity in substantia nigra. Related to Figure 5. *A*, 40x magnification of DAB immunostaining for CD68 in substantia nigra sections from Littermate control and MitoPark mice treated with vehicle or Mito-apo. Scale bar = 100 µm, inset is 2x of original image.

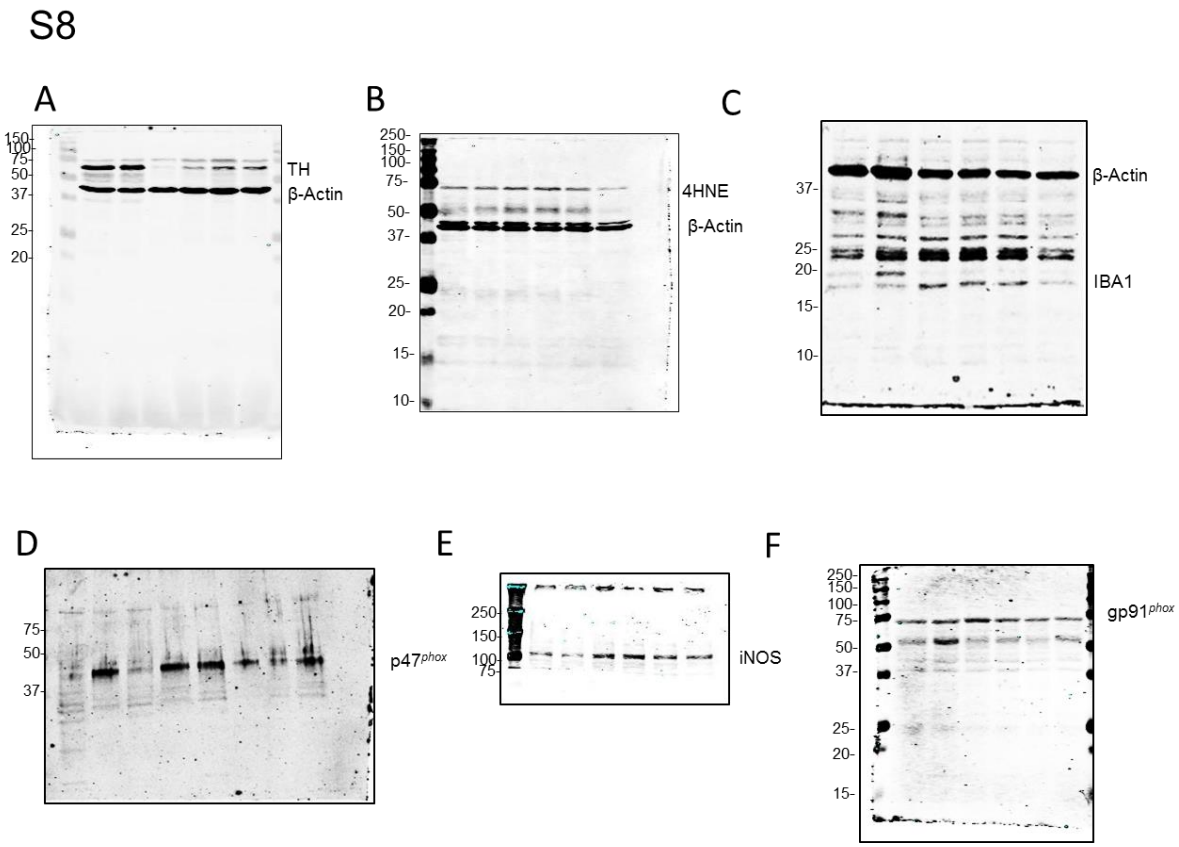

Fig S8. Full Western blotting images. Full images of cropped Western blot bands corresponding to Figure 3C (*A*), 4F (*B)*, 5A (*C*), 6A *(D*), 7B (*E*), and 7D (*F*).

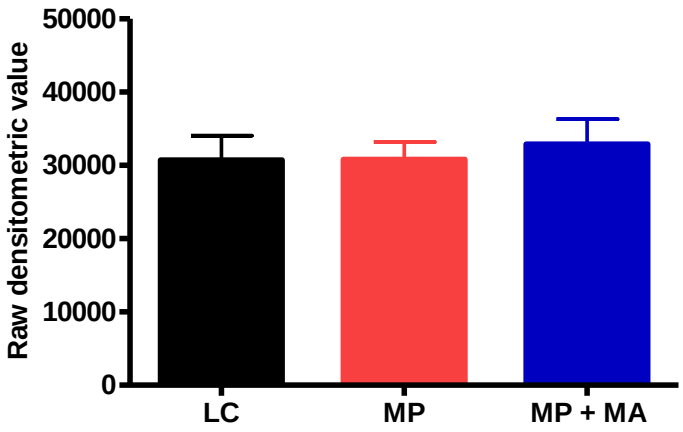

Figure S9. Densitometric analysis for β-actin. No significant difference found between treatment groups for β-actin by one-way ANOVA, confirming its usefulness as a protein loading marker in this study.

CHAPTER 5

PROKINETICIN-2 ATTENUATES ALTERED NEUROGENESIS AND NONMOTOR DEFICITS IN PARKINSON'S DISEASE MODELS

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Langley, Monica R.; Neal, Matt; Sarkar, Souvarish; Luo, Dan; Kondru, Naveen; Palanisamy, Bharathi N; Berg, Carrie; Jin, Huajun; Anantharam, Vellareddy; Kanthasamy, Arthi; Kanthasamy, Anumantha G.

Biomedical Sciences, Iowa State University, Ames, IA

*To whom correspondence should be addressed: Dept. of Biomedical Sciences, Iowa State University, 2062 College of Veterinary Medicine Building, Ames, IA 50011, USA. Tel: 1-515-294-2516; Fax: 1-515-294-2315; Email: akanthas@iastate.edu

Abstract

Recently, we showed that a secreted protein involved in olfactory bulb neurogenesis and circadian rhythms, Prokineticin 2 (PK2), plays a novel compensatory protective role in nigral dopaminergic neurodegeneration in Parkinson's disease (PD), yet the exact mechanism of the neuroprotective response remains elusive. We hypothesize that PK2 rescues impaired neurogenesis in PD models by promoting differentiation, proliferation, or migration of neural stem cells in neurogenic brain regions. First, we observed that recombinant PK2 treatment of organotypic brain slices increased the number of $BrdU^+$ cells in the subventricular zone (SVZ), suggesting a stem cell proliferative action for PK2. Also, lentivirus-mediated overexpression of PK2 significantly increased $BrdU/Tuj1^+$ neurons in brain slices containing the SVZ or dentate gyrus (DG), providing evidence of increased neuronal differentiation in control and MPP^+ neurotoxin-treated slices. Next, we extended our studies to MitoPark mice, a transgenic mouse model of PD that recapitulates key features of the disease including progressive neurodegeneration and motor deficits. We have recently shown that these animals exhibit several clinically relevant nonmotor symptoms including olfactory dysfunction, learning and memory deficits, and neuropsychological problems. In both animal models and human PD, reduced dopamine levels correlate with a reduced proliferation of cells in neurogenic regions of the brain, which is thought to contribute to the nonmotor symptoms observed in PD. Immunohistological staining revealed fewer proliferating cells in the SVZ and DG of aged MitoPark mice. Importantly, PK2 transgene delivery by adeno-associated virus (AAV) to striatal regions enhanced SVZ neurogenesis in MitoPark mice and attenuated the corresponding nonmotor symptoms at ages 16 and 24 wks as measured by the novel scent test and Forced Swim test. Furthermore, PK2-GFP AAV resulted in increased

expression of Jumonji Domain Containing protein 3 (JMJD3/Kdm6b), a histone lysine demethylase required for neuronal commitment in the SVZ when compared to GFP AAV-injected mice. Similar results were found in bone marrow derived stem cells treated with the PKR1 agonist IS20. Overall, these novel findings suggest that the development of nonmotor symptoms in MitoPark mice coincides with impaired adult neurogenesis, and that PK2 overexpression can restore neurogenesis by promoting proliferation and differentiation of neural stem cells through upregulation of JMJD3.

Introduction

Caused by complicated interactions between genetics and environmental factors, Parkinson's disease (PD) is a chronic, progressive neurodegenerative condition affecting nearly 10 million people worldwide. PD neuropathological features include a loss of dopaminergic neurons in the substantia nigra (SN) of the brain, leading to a functional loss of dopamine, and accumulations of abnormal α-synuclein protein aggregates. Although nonmotor deficits may occur decades prior to the onset of motor deficits, clinical diagnosis of PD is still largely based on cardinal motor symptoms such as bradykinesia, tremor, rigidity, and postural instability.

Previously, it was a long held belief that the mammalian brain did not grow new neurons, but in the past decade much research has been done to clarify the locations and functions of adult born neurons in the brain[1,2,3,4]. It is now understood that the adult mammalian brain continuously makes new neural stem cells (NSCs) in the subventricular zone (SVZ), which lines the lateral ventricles, and in the subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampal formation[5]. Adult NSCs have the capacity to differentiate into neurons, allowing for adaptation to stimuli in our environment[6,7,8]. In both animal models and human PD, reduced dopamine levels correlate with a reduced proliferation of cells in neurogenic regions of the brain, which is thought to contribute to the nonmotor symptoms observed in PD[9,10,11]. Although animal models and human post-mortem studies do indicate that adult neurogenesis is affected in PD, the exact mechanism of the changes and the correlation with nonmotor symptoms has not been directly explored[12]. Moreover, aging is associated with a reduction in adult neurogenesis and the accompanying cognitive deterioration in the elderly, and is a key risk factor for neurodegenerative

diseases[13,14]. Recently, we have verified the presence of nonmotor symptoms are present in MitoPark mice, a transgenic model that recapitulates key features of PD such as progressive neurodegeneration, levodopa- responsive motor deficits, and neuronal protein inclusions[15,16,17].

Prokineticin 2 (PK2) is a secreted protein with two differentially expressed G-protein coupled receptors (GPCRs), giving rise to diverse biological functions including olfactory bulb neurogenesis, circadian rhythms, gastrointestinal motility, and nociception[18,19,20]. As the mammalian homologues to MIT-1 and Bv8, respectively, prokineticin-1 (PK1) and PK2 proteins were first discovered as potent mediators of gastrointestinal motility found in black mamba snake venom and toad skin[20]. In the brain, PK2 is known to regulate olfactory bulb neurogenesis by serving as a chemoattractant and detachment signal for NPCs in the SVZ and OB, respectively[21]. Its receptor PKR2 is found in the SVZ, OB, RMS, and DG[21]. Additionally, in hypothalamic neurons, PK2 plays a role in circadian rhythms and food intake, while in gonadotropin-releasing hormone neurons, PK2 mediates their migration from the nose to the brain during development[22,23,24.] Basic helix-loop-helix transcription factors, such as MASH1 and CLOCK, or hypoxia can regulate PK2 expression in these distinct biological processes[25,26]. Interestingly, a recent study showed that PK2 expression was associated with cell proliferation and migration of new neurons toward the injury site following brain injury in a zebrafish model[27].

We have recently shown that PK2 expression is upregulated in the substantia nigra (SN) following exposure to toxicants and seems to play a compensatory role in early stages of nigral dopaminergic neurodegeneration[28]. In MitoPark mice, we have seen that PK2 protein expression in the SN is higher at 8 and 12 weeks of age, before major motor deficits

appear[28]. Our group has also shown that PK2 increases certain neurotrophic factors which could potentially be used to aid the migration of neural progenitor cells (NPCs) to the site of injury or to increase differentiation[29]. Therefore, we explored the regulatory functions of PK2 during altered neurogenesis in animal and cell culture models of PD by identifying the genotypic and phenotypic effects of PK2 overexpression in adult neural stem cells (aNSCs) under normal and pathological conditions. We also evaluated the protective ability of PK2 against nonmotor behavioral symptoms present in MitoPark mice. Overall, these studies should provide convincing evidence that the development of nonmotor symptoms in MitoPark mice coincides with impaired adult neurogenesis, and that PK2 can regulate neurogenesis by promoting the proliferation and differentiation of NSCs. Furthermore, we have identified key signaling pathways associated with PK2's effects on neurogenesis.

Results

Recombinant Prokineticin 2 increases neural stem cell proliferation in SVZ slice culture

Our recent publication determined that PK2 expression increased in dopaminergic neurons in mouse models as well as human PD, elucidating a novel role of PK2 in PD pathogenesis[28]. In one of these models, the MitoPark mouse, we have also identified clinically relevant nonmotor symptoms such as olfactory deficits, neuropsychiatric effects, and cognitive dysfunction[15]. Reduced adult neurogenesis is thought to contribute to nonmotor symptoms in PD patients [10,12,30]. Although some key mechanisms were identified that contribute to the neuroprotective effects in PD models, PK2s role in neurogenic regions of the brain was not explored.

Expanded neurosphere cultures showing increased potential for generating neurons by expressing various pro-neural genes have failed to maintain a substantial number of neurons once implanted[31,32]. Given the strong impact of environment and a discrepancy between *in vitro* and *in vivo* differentiation potential, we first used brain slices to assess the role of PK2 on stem cell proliferation and differentiation. Brain slices containing the subventricular zone (SVZ, Figure 1A) and dentate gyrus (DG, Figure 1B) were collected from day 9- to 12- day old mouse pups as previously described[33, 34], and maintained in culture for up to 2 weeks**.** Slices were incubated with bromodeoxyuridine (BrdU, 10µM) for 24 h after a 12 h treatment with recombinant PK2. Treatment with 50nM recombinant PK2 increased cell proliferation in SVZ slices as determined by $BrdU^+$ cells (Figure 1C), suggesting that morphology is maintained in cultured brain slices and recombinant PK2 protein induces a stem cell proliferative effect in the SVZ.

PKR1 agonist, IS20, increases proliferation and differentiation of NSCs

Given the potential impact of signaling from neighboring cells on differentiation potential, we cultured brain slices to assess the role of PK2 on NSC proliferation. Recently, a non-peptide agonist for the PKR1 receptor, referred to as IS20, was discovered and used therapeutically following myocardial infarction in mice[35]. Treatment with the PK2 receptor agonist IS20 increased the numbers of $BrdU^+/Tuj1^+$ (Figure 2B,D) and $BrdU^+/GFAP^+$ cells in SVZ slices (Figure 2 A,D). Co-labeled cells from z-stacked confocal images were quantified using the "Coloc 2" function (Figure 2C, D) in Image J. Overall, IS20 treatment increased proliferation of NSCs in the SVZ of brain slices and promoted their differentiation to a neuronal phenotype.

Lentivirus-mediated overexpression of PK2 increases proliferation and differentiation of NSCs in slice cultures

Next, organotypic brain slices were treated with 5µl of GFP or PK2-GFP lenti-virus prior to culturing for one week (Figure 3A). Slices were then treated with 30 µM MPP+ or vehicle and cultured for an additional 24 h prior to fixation and immunostaining procedures (Figure 3A). PK2 lentiviral treatment increased the total number of $BrdU^+$ proliferating cells and $Tuj1^+$ immature neurons in MPP^+-treated slices as determined by IHC staining and confocal imaging (Figure 3B). PK2 lentiviral expression also increased BrdU and Tuj1 immunoreactivity in untreated control SVZ and DG slices (Figure 3 B, C). These findings provide evidence for PK2 lentiviral-based overexpression inducing both proliferation of NPCs and their differentiation to an immature neuronal phenotype in an *ex vivo* mixed culture system.

Altered neurogenesis in the SVZ and DG of aged MitoPark mice

Our recent publications revealed that PK2 is upregulated in early dopaminergic neurodegeneration in MitoPark mice before clinically relevant nonmotor symptoms are present[15,28]. The SVZ-RMS-OB pathway is responsible for olfactory function, while hippocampal neurogenesis in the SGZ is linked to both depression-like behavior and cognition. Therefore, we injected MitoPark and control mice with BrdU to see if neurogenesis was affected in this model of chronic, progressive dopaminergic neurodegeneration. Littermate control and MitoPark mice were injected i.p. with 100 mg/kg BrdU daily for 3 days and sacrificed 12 h past the last injection as shown in the treatment paradigm (Figure 4A). DAB-IHC for anti-BrdU was performed on 30-µm sections containing the SVZ (Figure 4B) and SGZ (Figure 4C) to label proliferative cells, counterstained with hematoxylin. MitoPark mice had far fewer $BrdU^+$ cells in the SVZ in contrast to littermate control mice at ages 16 and 24 wks (Figure 4B). Similarly, a decrease in $BrdU^+$ cells is observed in the SGZ in hippocampal sections from 16- to 24-wk-old MitoPark mice (Figure 4C). Overall, BrdU staining revealed fewer proliferating cells in both the SVZ and SGZ of aged MitoPark mice, at ages paralleling the nonmotor deficits observed in this model.

Stereotaxic PK2-AAV administration enhances neurogenesis and attenuates nonmotor deficits in MitoPark mice

Considering that PK2 overexpression increased neurogenesis in our *in vitro* and *ex vivo* systems, we next extended our studies to MitoPark mice, which showed impaired neurogenesis from 16 wks onward. MitoPark mice were stereotaxically injected with 5µl of either GFP or PK2-GFP AAV 2/5 into the striatum at 8wks of age, prior to the onset of motor

deficits. Mice were sacrificed at 24wks of age (Figure 5A), when stem cell proliferation and nonmotor deficits were most affected. Immunohistochemistry on 24 wk tissues revealed an increase in $Ki67^{+}$ proliferating cells in the SVZ of PK2-GFP AAV mice when compared to GFP-AAV injected mice and abundant viral GFP expression in both groups (Figure 5B). Furthermore, occupancy plots of the animal's head during 3 min novel scent test trials show increased preference for the scented region in PK2-GFP AAV mice when compared to GFP AAV controls (Figure 5C). PK2-GFP AAV mice spent significantly more time sniffing the scented zone than the GFP AAV mice (Figure 5D). Moreover, PK2-GFP AAV injected mice spent significantly less time immobile during the Porsolt forced swim test (Figure 5E), indicating reversal of the depressive-like phenotype in aged MitoPark mice. Although increased PK2 expression helped restore neurogenesis and attenuate nonmotor symptoms in MitoPark mice, no significant change in endogenous PK2 expression was found in the hippocampus or olfactory bulb of MitoPark mice when compared to littermate controls at 8 or 17 wks of age (Supplemental Figure 5).

PK2 overexpression increases EGR1, BDNF, and JMJD3 in the SVZ of MitoPark mice

In animal models and human PD, dopaminergic deafferentation results in dysregulated neurogenesis [12,30,36,37]. Therefore, we were interested in seeing if PK2's neuroprotective role extended to stem cells. Bone marrow derived stem cells (BMSCs) can be induced to differentiate into neural cells when cultured in the presence of neurotrophic factors [38-40]. In mouse BMSCs we found that PKR2 receptor expression was significantly higher than PKR1 expression as determined by qPCR (Supplemental Figure S1). Other papers have shown that PKR2 is expressed in the SVZ-RMS-OB pathways[21,25], indicating it as the primary receptor involved during PK2's functions in normal neurogenesis. Both ERK

and CREB signaling have been indicated in regulation of neurogenesis and PK2's neuroprotective effects, revealing a potential mechanism for PK2s role in neurogenesis[41,42]. The histone demethylase JMJD3 controls the expression of key regulators of neurogenesis and is considered essential to neuronal differentiation[43,44]. Exogenous expression of JMJD3 has been shown to increase in early stages of neural differentiation[45]. JMJD3 is also upregulated in glial cells, resulting in attenuation of neuroinflammation [29]. Here, we show that JMJD3 mRNA expression is increased following 8hr PK2 treatment in BMSCs (Figure 6A). Neurotrophic factor BDNF also had higher mRNA expression after PK2 treatment (Figure 6B). Previous research in our lab identified another neurotrophic factor, GDNF, to be increased following PK2 exposure in various cell types[29]. Also, we have shown that BDNF and CREB phosphorylation levels decrease, while the oxidative stress marker 4-HNE is increases in the hippocampus of MitoPark mice[15].

Next, we used IHC to check for expression of proteins involved in neurogenesis in SVZ brain sections from GFP or PK2-GFP AAV injected mice. First, we saw an increase in EGR1 (Figure 6C), which controls maturation and integration of new neurons as a result of learning behavior[46]. Furthermore, we saw an increase in JMJD3 protein expression by immunohistochemistry (Figure 6D), which is required for neural commitment of cells, in the subventricular zone. A similar increase in JMJD3 was also observed in the dentate gyrus of the hippocampal formation and olfactory bulb (Supplemental Figure 3). Confirming our findings in BMSCs, we also saw increased BDNF expression in the hippocampus and striatum in the PK2-GFP-AAV group compared to the GFP-AAV injected MitoPark mice (Supplementary Figure 4). These results suggest that PK2 may help induce neuronal differentiation by increasing the expression of key factors mediating neurogenesis.

IS20 increases the histone demethylase JMJD3 in BMSCs

Recombinant PK2 induced about a 3-fold increase in JMJD3, and stereotaxic administration of PK2-GFP AAV increased JMJD3 in brain tissues. Next, we wanted to compare recombinant PK2 data to the recently developed PK2 receptor pharmacological agonist, IS20[35]. Although IS20 was not able to significantly increase JMJD3 expression at the low concentrations used for recombinant PK2, 8 hr treatment of 30μM IS20 significantly increased JMJD3 gene and protein expression in BMSCs, as determined by qPCR and immunocytochemistry, respectively (Figure 7A, D). This difference is likely due to IS20's specificity to the PKR1 receptor, while PKR2 is predominantly expressed in these cells and neurogenic brain regions (Supplemental Figure 1).

Discussion

Because current treatments for PD do not halt or decelerate the neurodegenerative process, alternative treatment approaches such as cell replacement therapies have been explored as an option despite known difficulties with grafting. Ethical concerns, immunoresistance, and limited availability present major problems in using fetal tissue as a source for dopaminergic cell replacement strategies in PD[49,50]. Although patient-derived expanded neurosphere cell cultures may alleviate these issues, thus far only poor yields of neurons have been achieved by researchers engrafting into non-neurogenic regions of the brain such as the striatum[51,52,53]. Our novel results suggest that PK2 overexpression may enhance differentiation and proliferation of adult NSCs, which could potentially attenuate alterations in neurogenesis associated with PD. Our previous work shows that PK2 overexpression can provide neuroprotection from neuroinflammation and neurodegeneration in the MPTP model, and that a PK2 antagonist worsens disease progression in the model[28]. Also, we recently revealed that the MitoPark model showed nonmotor symptoms associated with PD such as olfactory dysfunction, learning and memory deficits, and neuropsychological problems[15]. We have now provided evidence to show that PK2 overexpression can attenuate changes in neurogenesis and reverse clinically relevant nonmotor symptoms in the MitoPark mouse model.

Importantly, in addition to BrdU, Ki67 was also used for this study. This is critical because BrdU intercalates into DNA, so DNA damage in addition to cell proliferation could result in BrdU staining[54]. Findings with BrdU and Ki67 were similar, suggesting that staining was due to proliferation and not DNA damage present due to the neurodegeneration found in other brain regions in this model. Studying neurogenesis by these methods is essential before

trying to elucidate signaling pathways associated, because decreases in neurogenesis due to age and other factors is not always associated with reduced expression of genes important for proliferation and differentiation in neurogenic regions of the brain[55].

Recent work in our lab demonstrated that exogenously added PK2 can induce an alternative activation phenotype of mouse primary microglia through the STAT6-JMJD3-Irf4 signaling pathway via the PKR1 receptor[29]. JMJD3 is thought to play a role in microglia activation and neuroinflammation in PD and AD[56,57]. Due to its role in polarization of microglia cells to the M2 phenotype via STAT6[44], JMJD3 may also indirectly contribute to increased neurogenesis by suppressing microglia activation[57]. This should be considered in future studies, as many papers have shown neuroinflammation to inversely correlate with neurogenesis[58,59,60]. STAT1 and STAT3 transcriptionally regulate JMJD3 in regards to inflammatory responses[61]. STAT3 inhibition also leads to permanent neurosphere formation defects[62]. Future studies should direct attention to the possibility of PK2 interactions with EGR1 and STAT3.

Our *in vivo* and *ex vivo* model systems used in this paper included neighboring cell types, so additional studies in primary isolated aNSCs would be beneficial to explore the direct role of JMJD3 on neural stem cell differentiation. Another paper has shown JMJD3 to be essential for vitamin C's role in neural stem cell differentiation in culture[63], suggesting that at least part of the effect on NSCs is direct. These coordinated effects reveal PK2 overexpression, and its downstream effects, to be a promising new target for promoting neural repair through resident stem cells.

PK2 has already been shown to be involved in olfactory bulb neurogenesis but has not been linked to JMJD3 or pathological states in its neurogenic role[21,43]. EGR1 is essential

for the maturation and functional integration of adult hippocampal neurons during learning tasks as evidenced by knockout studies[46]. Egr-1 is induced under hypoxia/hypoglycemia conditions, leading to increased EGF-R levels and promoting the regenerative response observed in neural stem and progenitor cells in the SVZ[64]. Moreover, a very recent study identified EGR1 as a novel upstream target of JMJD3/Kdm6b, cooperatively regulating it along with CREB and AP-1[65,66,67]. Sustained ERK activity is essential for neuronal differentiation and can be induced by NGF; whereas transient ERK activity can be achieved through EGF[68]. Our recent publication shows that PK2 can upregulate ERK in neurons, contributing to its neuroprotective effect[28]. More advanced studies using ERK and PK2 specific inhibitors are needed to fully elucidate PK2s role in ERK signaling in neural stem cells.

Another possible regulator of JMJD3 is BDNF. Activity-related changes in BDNF, JMJD3, and PK2 have all been shown following seizure activity in the brain[69,70,71]. PK2 was also recently shown to increase in models of PD, epilepsy, and traumatic brain injury[28,27,71]. Interestingly, JMJD3 expression is known to be induced following neuronal activity, resulting in the expression of BDNF promotors[72]. While BDNF also induces JMJD3 transcription in cultured hippocampal neurons, JMJD3/Kdm6b is not necessary for BDNF-mediated survival in neurons[73]. Our previous publication showed PGC1α was upregulated by PK2 in regards to mitochondrial biogenesis in neurons[28]. Recent research also suggests PGC1α's role in the CREB/BDNF positive feedback elicited by exercise, leading to neuroprotection and neurogenesis[74].

IS20, a novel non-peptide prokineticin receptor agonist, was recently used therapeutically in a mouse model of myocardial infarction where it was shown to be highly

selective for PKR1 [35]. Gasser et al were able to show that IS20 increased angiogenesis and proliferation of cardiomyocytes and epicardial progenitor cells after IS20 treatment following myocardial infarction [35]. Recent work in our lab demonstrated that exogenously added recombinant PK2 can induce an alternative activation phenotype of mouse primary microglial cell cultures through the STAT6-JMJD3-Irf4 signaling pathway via the PKR1 receptor[29].

First, we found that PK2 treatment or PK2 lentiviral infection in brain slices containing both the striatum and SVZ increased proliferation of neural precursor cells and their differentiation to a neuronal phenotype in an *ex vivo* mixed cell culture system. We also discovered that MitoPark mice, a chronic progressive model of dopaminergic neurodegeneration, exhibit altered neurogenesis in the SVZ and DG at ages correlating to the onset of nonmotor symptoms clinically relevant to PD. Next, we found that PK2 transgene delivery by AAV in the striatum enhanced neurogenesis and attenuated corresponding nonmotor symptoms in aged MitoPark mice. Altogether, it seems PK2 plays a neuroprotective role in PD by regulating adult neurogenesis through JMJD3, making it a promising new therapeutic target for gene therapy or for the development of novel CNS bioavailable PKR1 or PKR2 agonists.

Methods

Chemicals

5-Bromo-2′-deoxyuridine (5-BrdU), 3,39-diamino-benzidine (DAB), sodium borate, HCl, sodium citrate, paraformaldehyde, and anti-β-actin antibody were purchased from Sigma-Aldrich (St Louis, MO). Cell culture media, trypsin, Horse serum, penicillin, streptomycin, low-melting agarose, Halt™ Protease and Phosphatase Inhibitor Cocktail, and Alexa Fluor 680 conjugated goat anti-mouse IgG was purchased from Thermo Fisher (Carlsbad, CA). Goat anti-rabbit IR800 conjugated IgG was obtained from Rockland Immunochemicals (Gilbertsville, PA). Millicell 6-well plate inserts were obtained from EMD Millipore (Billerica, MA).

Cell culture

Bone marrow derived stromal cells (BMSCs) isolated from mice were kindly provided by Dr. Don Sakaguchi's lab at Iowa State University[75]. Cells were cultured in T75 flasks seeded with 2 million cells and grown in Iscove's modified Dulbecco medium containing 10% fetal calf serum, 10% horse serum, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 0.25µg/ml Amphotericin B, and 2 mM L-glutamine. Cells were treated with IS20 (10 or 30µM) for 6-8hrs prior to RNA isolation for qPCR experiments and 10 hr for immunocytochemistry.

Brain slice culture and treatments

All experiments involving animals followed the protocols approved by Iowa State University's Institutional Animal Care and Use Committee (IACUC). Organotypic brain slices containing SVZ or DG from 9-12 day old pups were collected at 350 µm intervals using a vibratome (Compresstome™ VF-300, Precisionary Instruments) as previously

described[34,33]. In brief, the brain was oriented in the sagittal plane in the Compresstome's specimen tube and solidified in 2% low-melting-point agarose by clasping the specimen tube with a chilling block. Next the specimen tube was inserted into the slicing reservoir filled with freshly prepared, ice-cold Gey's balanced salt solution (GBSSK). Slices were cut at 350-µm thickness with the blade set at a medium vibration speed and were collected at the specimen tube's outlet and transferred to another plate with fresh prefilled GBSSK. Later, the slices were washed twice and transferred to Millicell 6-well plate inserts (2 slices per insert) and were incubated in a humidified atmosphere of 5% CO2 at 37 °C. Culture media was exchanged every other day with fresh media until the pre-determined endpoints. Slice cultures were harvested at various endpoints by washing twice in 2 ml of ice-cold PBS. Slices were treated with GFP or PK2-GFP lenti-virus for 1 week prior to incubation with BrdU another 24 hrs. After exchanging media, slices were kept an additional 24 hrs prior to fixation and immunostaining procedures. Similarly, recombinant PK2 was incubated on slices at 50nM for 24hr and IS20 at 30µM for 24hrs prior to BrdU incubation step.

PK2-eGFP lentivirus production:

Lentiviral packaging and administration was performed as previously described[28]. Briefly: 293FT cells were plated in T75 flasks at 1.8 million per flask. The next day, cells were transfected with control eGFP lentiviral vector or PK2-eGFP lentiviral vector together with the Lentiviral Packaging Mix (Sigma) according to manufacturer's instructions. After 48 hours, supernatant were collected and spun down with ultra-centrifuge at 22k RPM. The resulting lentivirus pellet was resuspended in PBS and lentiviral titer was determined with the Lentivirus Titering Kit (Clonetech) according to manufacturer's instructions. Day 7 organotypic slices were transduced at approximately 10 MOI and cultured for one week.

Plasmid construction, generation and purification of rAAV:

PK2 mRNA sequence was obtained from NCBI and codon-optimized for expression in the mouse using the GeneArt Gene Synthesis service at LifeTechnologies. The finished plasmid was sent to the Viral Vector Core Facility at the University of Iowa for cloning. Briefly, Kpn1 and Xho1 restriction enzymes were used to subclone the codon-optimized PK2 sequence into the pFBAAV2/5CMVmcsBgHpA viral vector. To generate rAAV, the pFBAAV2/5CMV-PK2-eGFP-BgHpA or AAV2/5CMVeGFP control viral plasmid was transfected into SF-9 insect cells along with the helper plasmid pAAV-RC containing Rep/Cap sequences for viral packaging. The resulting packaged cis-acting AAV baculovirus carries the AAV expression cassette flanked by the AAV inverted terminal repeats (ITR). P1 baculovirus stock was used to amplify the P2 virus. For production of the virus, SF-9 cells were grown in bioreactors and infected with the P2 virus. Cells were harvested after signs of infection and collected in 50 mL tubes. Cells were pelleted and mechanically lysed with glass beads and a bead blender to release the virus. The lysate was treated with detergent to further lyse cells and dissociate virus from membranes and proteins. Benzonase was added to digest genomic, proviral, and plasmid DNA while leaving packaged viral DNA intact. The lysate was then clarified by centrifugation to obtain viral particles. Viral particles were purified using Iodixanol step gradients of 25%, 40%, and 60%, and full viral particles were then collected at the 40-60% interface. The viral particles were further purified using a Mustang Q Anion Exchange disk as per manufacturer's instructions. A qPCR assay verified AAV2/5CMV-PK2-eGFP-BgHpa viral genome titer of 2.98e+12.

Behavioral tests:

MitoPark mice were originally kindly provided from and generated by Dr. Nils-Goran Larson at the Karolinska Institue in Stockhom[16]. All mice for this study were bred, maintained, genotyped, and further characterized at Iowa State University. MitoPark mice (*DAT* $^{+/Cre}$*: TFAM* $^{LoxP/LoxP}$) and their littermate controls (*DAT* $^{+/+}$ *: TFAM* $^{+/LoxP}$) were fed *ad libitum* and housed in standard conditions approved and supervised by the Institutional Animal Care and Use Committee at Iowa State University.

The Porsolt "forced swim" test was used to assess depressive-like behavior in mice as previously described[15],[76],[77]. Briefly, Mice were placed in beaker (24 x 16cm) of water (up to 15cm, 25◦C), acclimated 2 min and recorded for 4 min. Unbiased motion monitoring for bouts of immobility was achieved using AnyMaze Software (Stoelting, Wooddale, US) based on 65% immobility for 2.5 seconds.

Social discrimination and novel scent tests were used to determine olfactory function as previously described[78],[79],[15]. Briefly, during a 3 min trial, time sniffing scented and non-scented zones were recorded using AMS. Scents used were lemon, peppermint, and vanilla for the novel scent test; whereas, social discrimination scented area contained bedding of opposite sex mice. The "unscented" zone was water or animals' own bedding, respectively.

Stereotaxic surgery:

C57BL/6 mice were anesthetized using a ketamine/xylazine mixture. The Angle 2 stereotaxic instrument was used with a 10-mL Hamilton syringe to inject the rAAV directly into the striatum at the following stereotaxic coordinates in relation to Bregma (mm): -2 ML, 0.5 AP, -4 DV. After drilling a hole in the skull, 5 µl of rAAV viral particles (~9e+12 total

viral particles) were injected into the brain. The animal was allowed to recover for four weeks to maximize viral gene expression before behavioral testing.

BrdU treatment paradigm:

Littermate control and MitoPark mice were injected intraperitoneally with 100 mg/kg BrdU daily for 3 days and sacrificed 12hours past the last injection as previously described[80]. For each age, 6-8 mice were used per group. Mouse brains were perfused with PFA, kept in sucrose, brain blocks made in OCT, and sections taken at 30 µm. Sections were treated with HCl to denature DNA prior to IHC to allow binding of BrdU antibody. DAB immunostaining was performed for BrdU (rat) and hematoxylin used as a counterstain for nuclei.

Immunocytochemistry and Immunohistochemistry

For immunostaining procedures, cells were plated at 40k cells in 6wells of 24wp overnight, followed by a 10hr PK2 treatment. For brain slices and 30µm sections of LC and MitoPark mice, immunohistochemistry was performed as previously described[28,34]. BrdU (10µM) was added to treatment media for 24hrs before fixing slices in 4% paraformaldehyde overnight. Prior to antigen retrieval in citrate buffer, DNA was denatured by keeping sections in 1N ice cold HCl 10 min, 2N HCl 37C, and 2 washes (15 min) borate buffer pH 8.5. Antibodies used- BrdU (rt) 1:250, GFAP (ms) 1:1000, Tuj1 (ms) 1:1000 and appropriate secondary. Hoechst 1:5000, 5 min. The culture membranes were carefully removed from the inserts with a scalpel and mounted directly on microscope slides, with membranes facing the slide, using Fluoromount mounting medium (Sigma). Bright field images were captured at 10x and 30x magnification using a SPOT digital camera and Nikon microscope. Confocal images taken using the Leica SP5 X confocal microscope system at the Confocal Microscopy

and Image Analysis Facility at Iowa State University. Image J software (NIH, Bethesda, MD) was used for quantification based on 6 Z-stacked images per group.

Diaminobenzidine (DAB) Immunostaining:

For DAB staining, Immunohistochemistry was performed as described above on 30µm sections containing SVZ or SGZ. However, a biotinylated secondary antibody followed by incubation with an avidin peroxidase solution was used to yield a brown stain after incubation with diaminobenzidine solution.

qPCR

Cells were seeded out at a density of 2 million cells into each T75 flask. RNA isolation was achieved through Trizol extraction as previously described[81]. RNA was quantified using Nanodrop spectrophotometer and 0.5ug RNA was converted to cDNA. Quantitative polymerase chain reaction (qPCR) was performed using either a Stratagene Mx3000 or QuantStudio 3 machine.

Statistics

All statistical analysis was done by one-way ANOVA or Student's t-test using Graph Pad Prism 7.0 software. Data are represented as the mean +/- SEM with $p < 0.05$ considered significant.

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Author Contributions

A.G.K., M.R.L., and M.L.N conceived and designed the experiments. M.R.L., M.L.N., S.S., D.L. and B.N.P. performed the experiments and. analyzed the data. N.K., C.B, and D.L. contributed reagents, cell lines, and mouse tissues. M.R.L., H.J, and V.A. wrote the paper. A.G.K. and A.K. and led the investigation, conceived the project, and wrote the manuscript. All authors reviewed and edited the manuscript.

Author Disclosure

A.G.K. and V.A. are shareholders of PK Biosciences Corporation (Ames, IA), which is interested in translating mechanistic studies into therapies targeting PK2 signaling. The remaining authors have no conflicts of interests.

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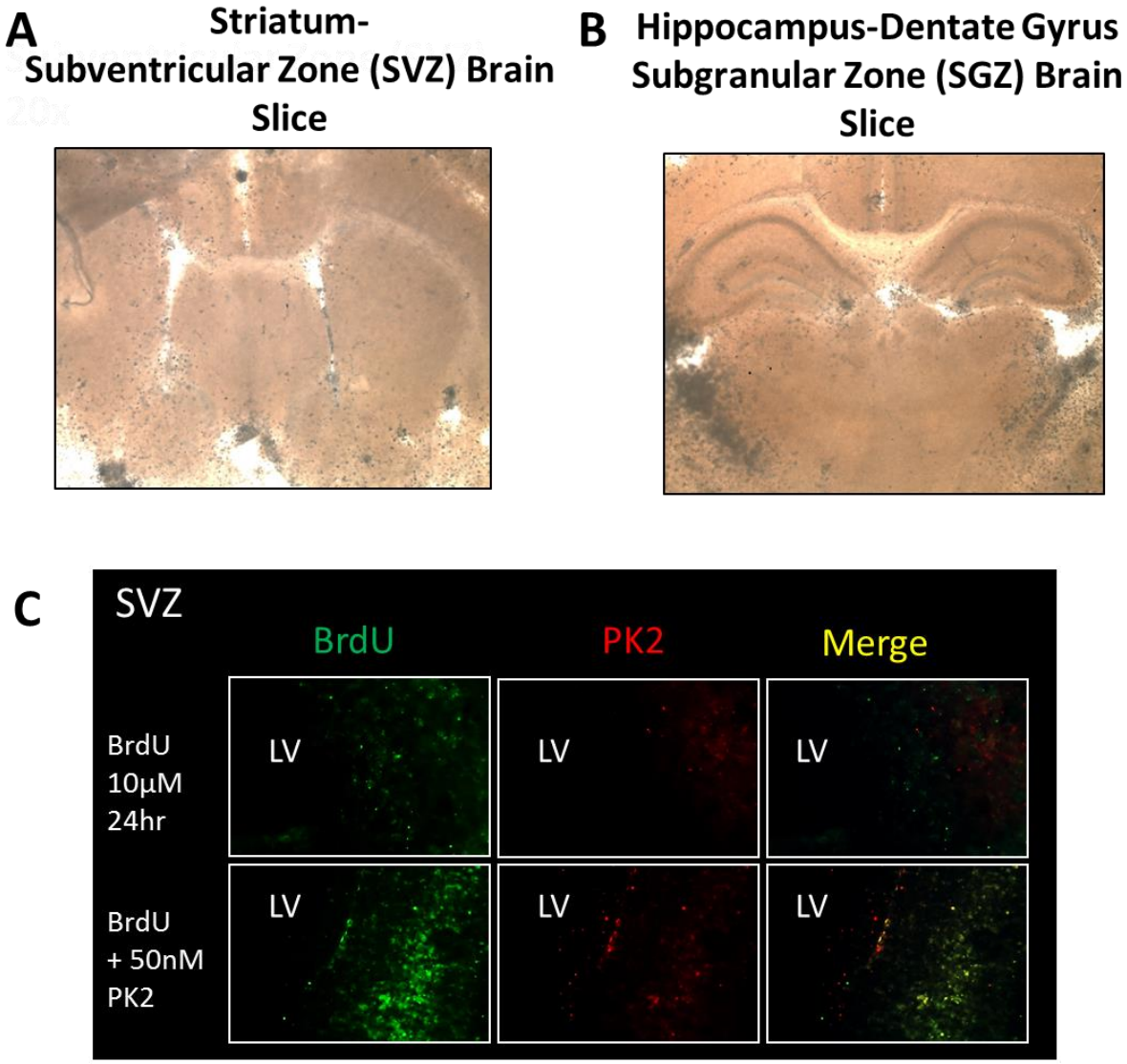

Figure 1. Recombinant Prokineticin-2 increases neural stem cell proliferation in SVZ slice culture. Photomicrographs using a 2x objective showing striatum/SVZ (*A*) and hippocampus/SGZ (*B*) cultured brain slices. *C*, Recombinant Prokineticin-2 (PK2, 50nM) treatment increases $BrdU^{+}$ (10uM, 24hr) proliferating cells in the SVZ slice culture.

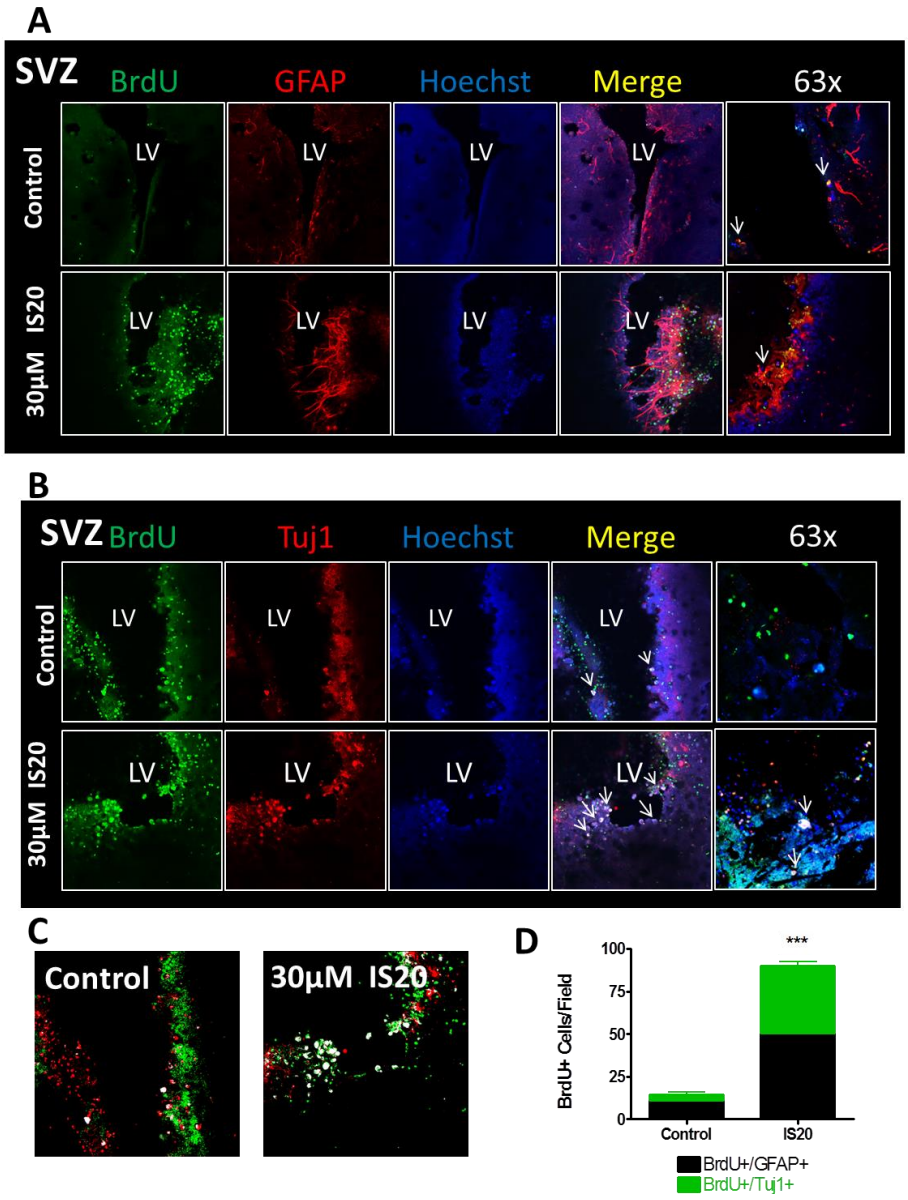

Figure 2. PKR1 agonist, IS20, increases proliferation and differentiation of NSCs in SVZ slice culture. Brain slice culture containing the SVZ shows increased proliferation as shown by increased BrdU+ cells. Of the neural stem cells identified by BrdU+ labeling, a portion are type B astrocytic stem cells (*A*), while others are type A/Tuj1^{+} neural progenitor cells (*B*). The "coloc2" function in ImageJ was used to quantify BrdU^{+}/GFAP^{+} and BrdU^{+}/Tuj1+ co-localized cells in the SVZ (*C, D*).

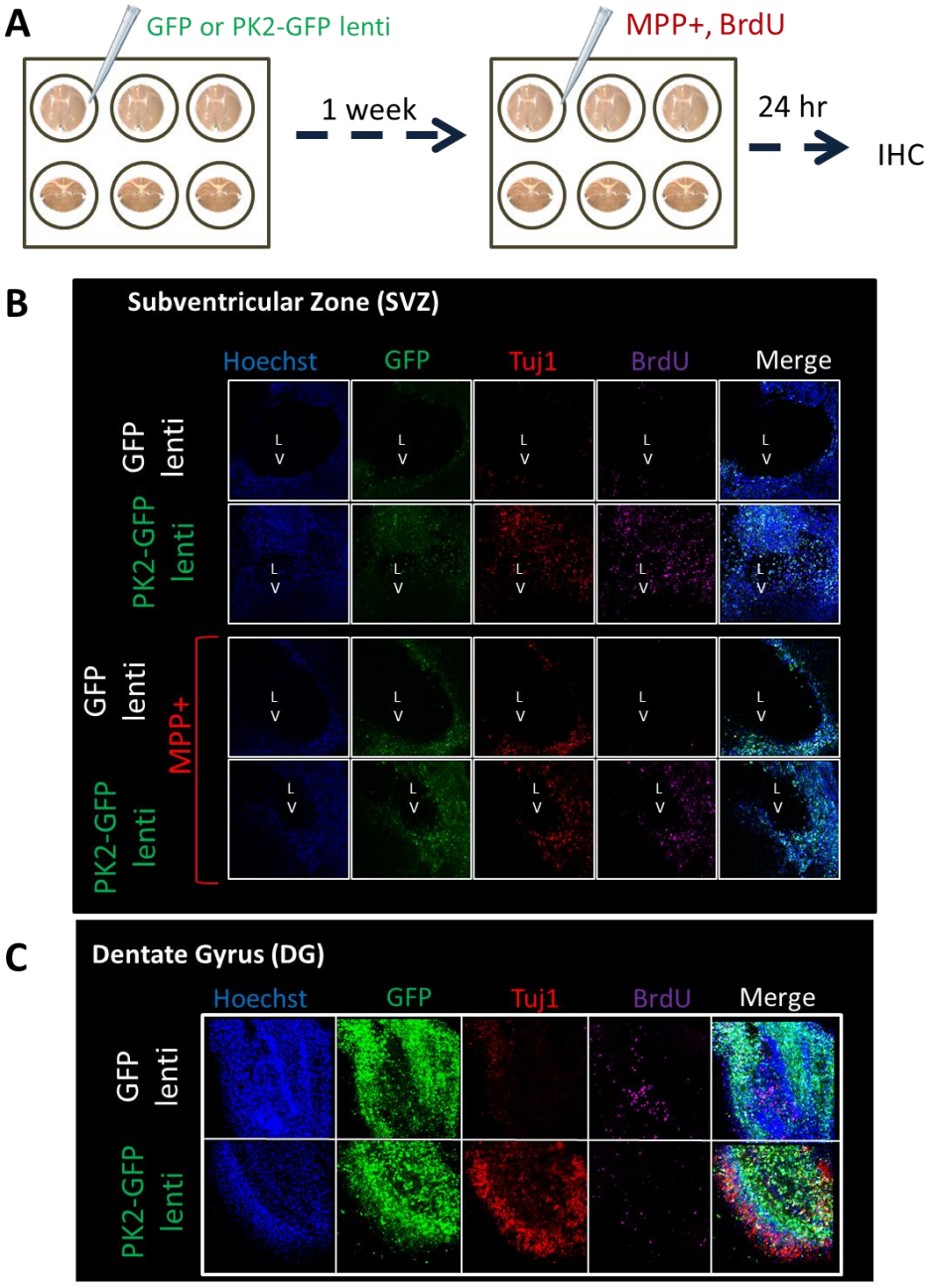

Figure 3. Lentivirus-mediated overexpression of PK2 increased proliferation and differentiation of NSCs in slice cultures. Organotypic brain slices were treated with 5µl of GFP or PK2-GFP lenti-virus prior to culturing for one week, then 30 µM MPP^+ or vehicle

and cultured for an additional 24 h prior to fixation and immunostaining procedures (*A*). Confocal imaging of immunohistochemistry shows PK2 lentiviral treatment increased the total number of $BrdU^+$ proliferating cells and $Tuj1^+$ immature neurons in MPP^+-treated slices (*B*). BrdU and Tuj1 immunoreactivity in DG slices *(C*).

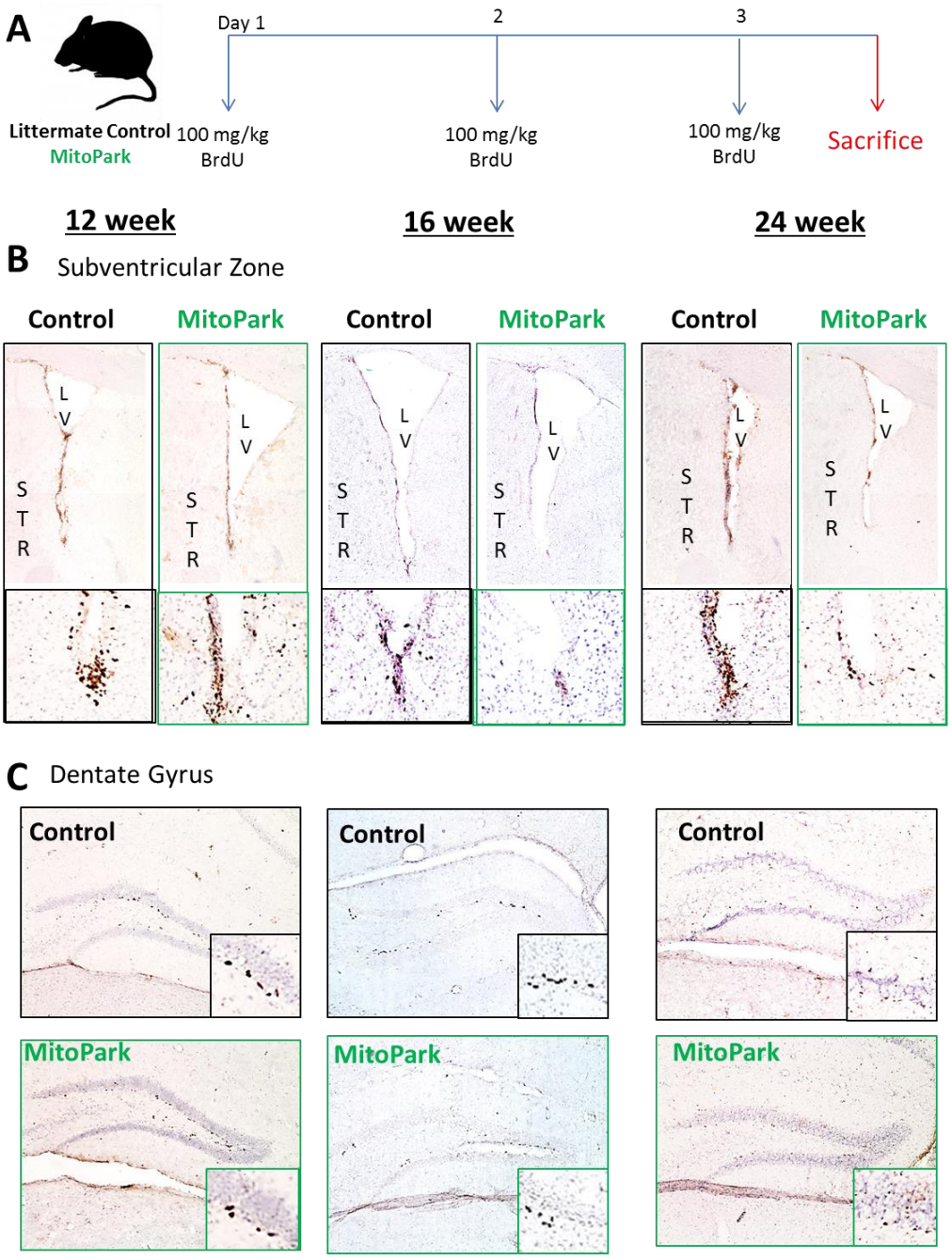

Figure 4. Altered neurogenesis in aged MitoPark mice. Littermate control and MitoPark mice were injected i.p. with 100 mg/kg BrdU daily for 3 days and sacrificed 12hours past the last injection as shown in the treatment paradigm at 12-, 16-, and 24-wks-of-age (*A*). DAB-IHC was performed on 30-micron sections containing SVZ (*B*) and SGZ (*C*) for anti-BrdU to

label proliferative cells, with hematoxylin used as a counterstain. Images were captured at 10x and 30x magnification. Control mice show a larger number of BrdU+ cells in the SVZ in contrast to MitoPark mice starting at 16 weeks of age (*B*). The same trend is observed in the SGZ in hippocampus sections (*C*).

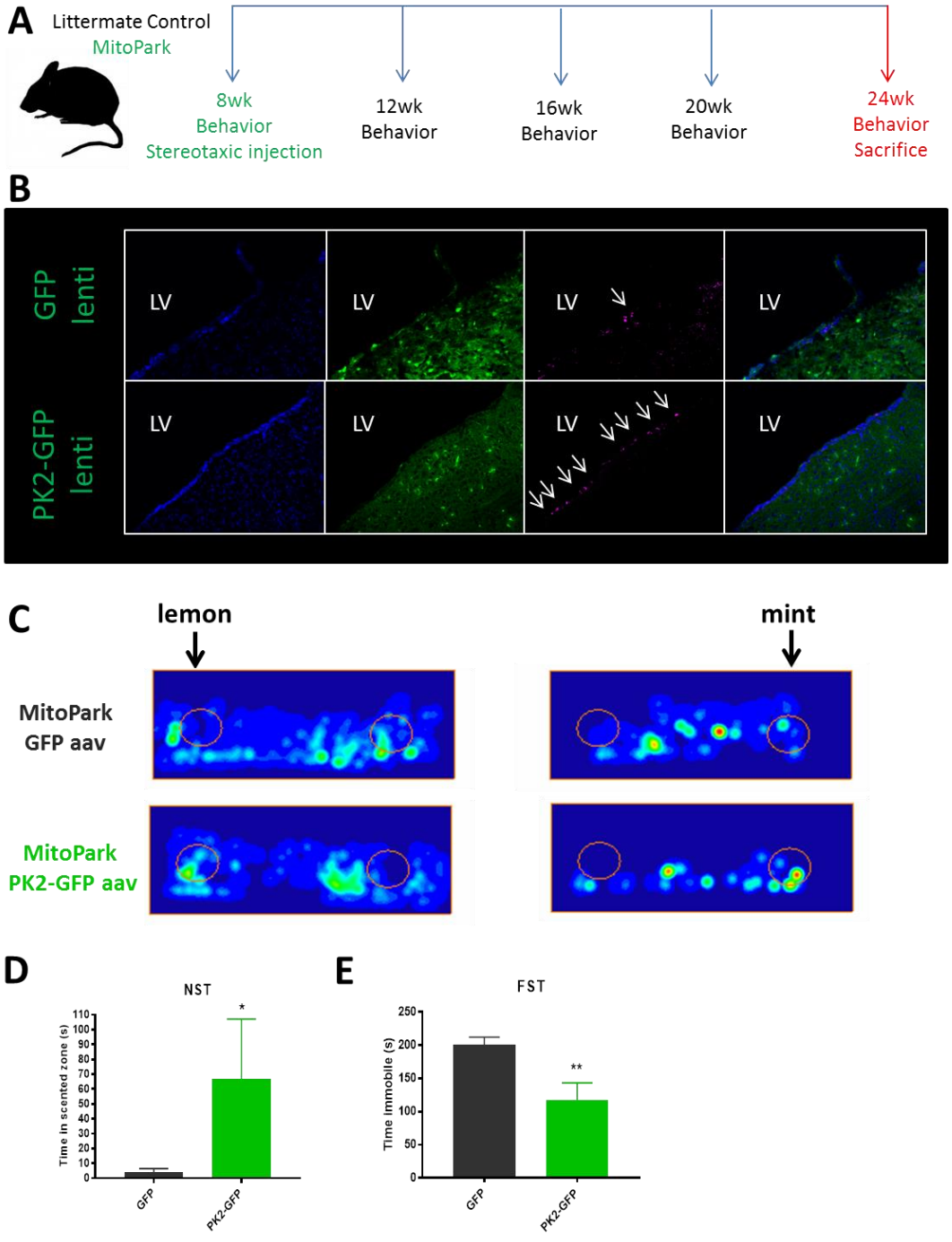

Figure 5. Stereotaxic PK2-aav administration enhances neurogenesis and attenuates nonmotor deficits in MitoPark mice. MitoPark mice were stereotaxically injected with GFP or PK2-GFP (*A*) adeno-associated virus (aav). IHC for Ki67 (purple, arrows) in SVZ

sections of 24 week old MitoPark mice reveal increased Ki67 staining with PK2-gfp versus GFP alone (*B*). At 24 weeks, the novel scent test (NST) (*C*, *D*) and forced swim test (*E*) reveal nonmotor symptoms, olfaction and depressive behavior, are improved in these mice. LV=lateral ventricle.

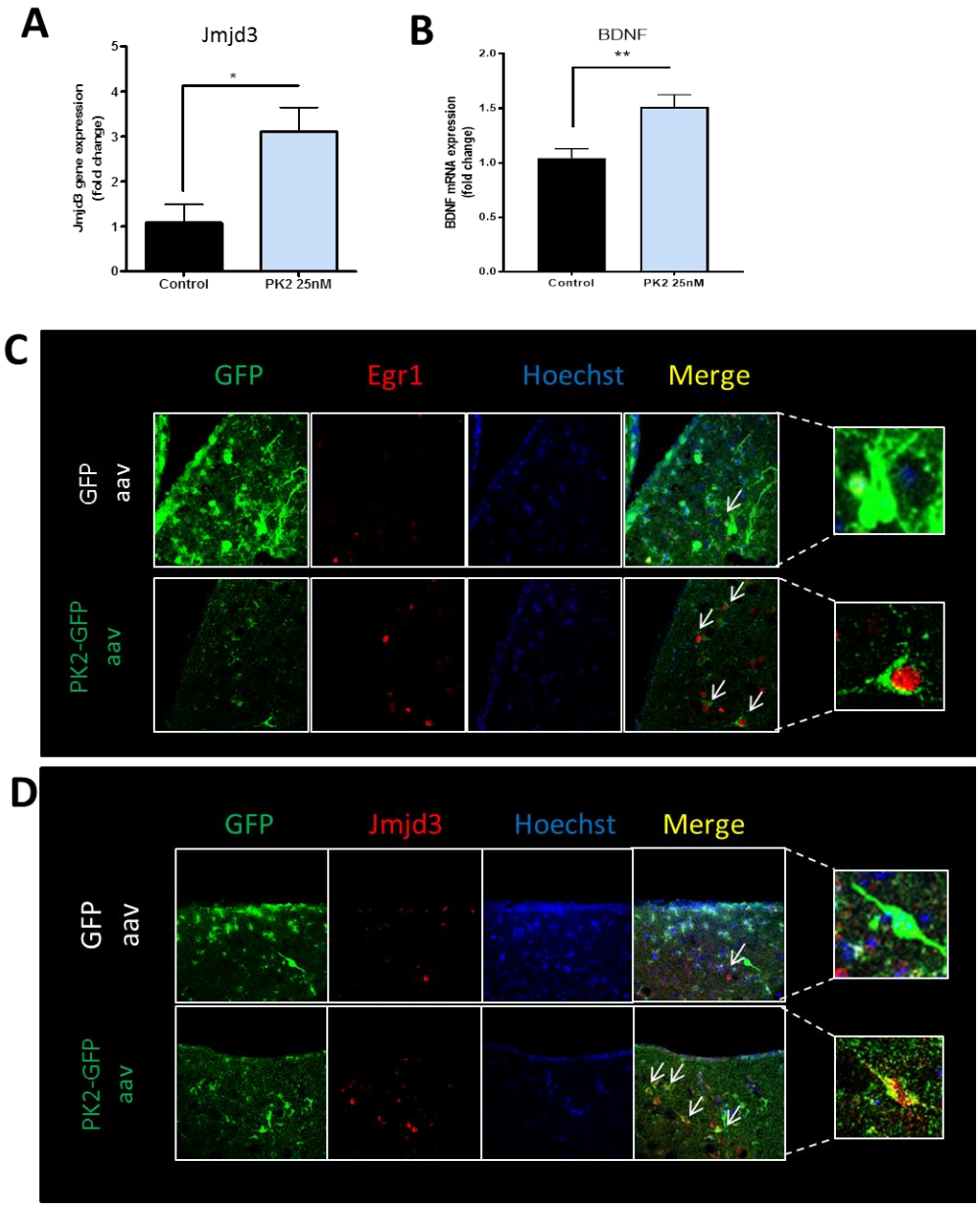

Figure 6. PK2 promotes neurogenesis by increasing the histone demethylase JMJD3. Recombinant PK2 increased required factors for neurogenesis in BMSCs (*A*, *B*). qPCR analysis for mRNA expression levels of JMJD3 (*A*) and BDNF (*B*). Confocal imaging of IHC for EGR1 (*C*) and JMJD3 (*D*) in MitoPark mice SVZ injected with GFP-aav or PK2-GFP-aav.

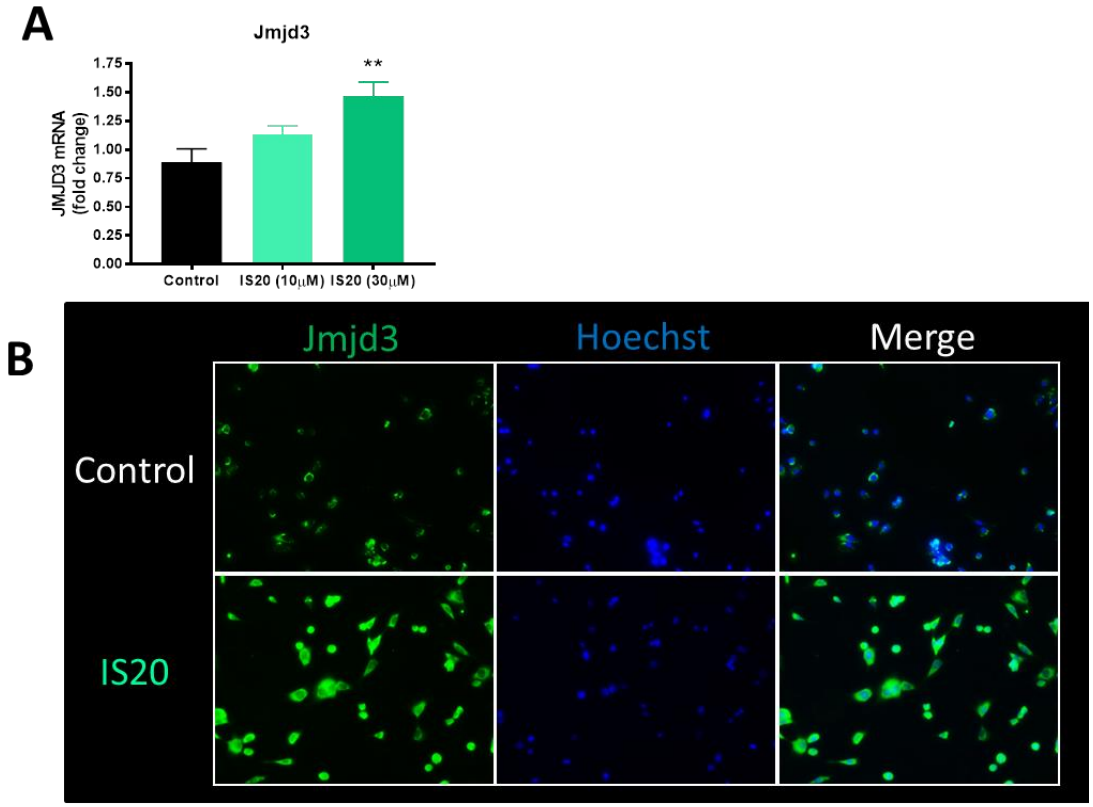

Figure 7. IS20 increases neurogenic markers in BMSCs. qPCR (A) and IHC (B) analysis showing increased mRNA and protein levels, respectively, of JMJD3 following IS20 (30μm) treatment.

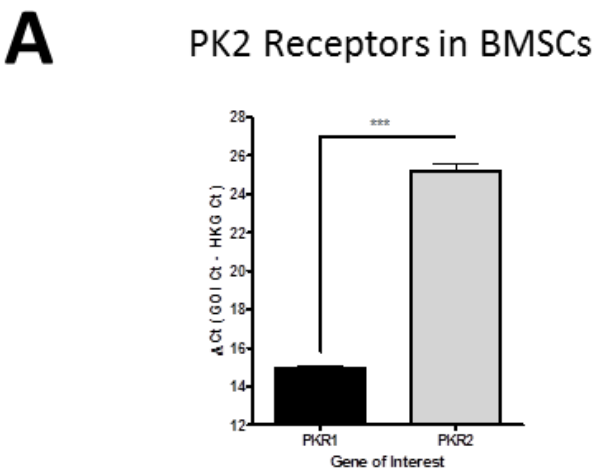

Figure S1. PK2 Receptors in bone marrow derived stromal cells (BMSCs). qPCR analysis (*A*) of prokineticin receptor levels in BMSCs.

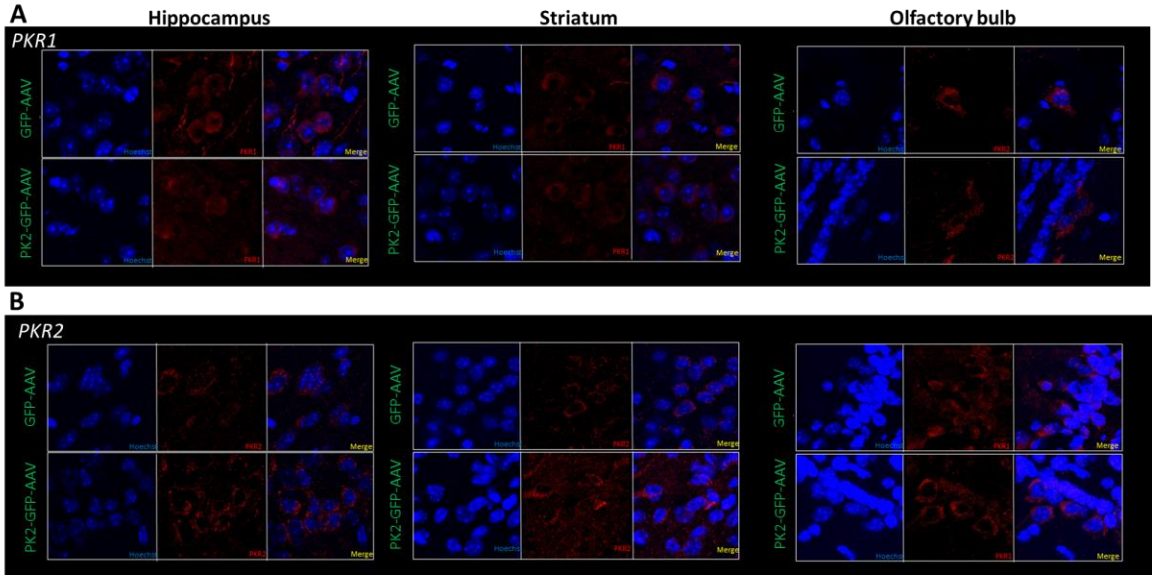

Figure S2. PK2 receptors in brain regions from MitoPark mice. Hippocampus, striatum, and olfactory bulb sections show no change in PKR1 expression (A) following PK2-GFP-AAV injection. PKR2 expression is slightly increased in the hippocampus and striatum of PK2-GFP-AAV-injected MitoPark mice when compared to GFP-AAV-injected MitoPark mice.

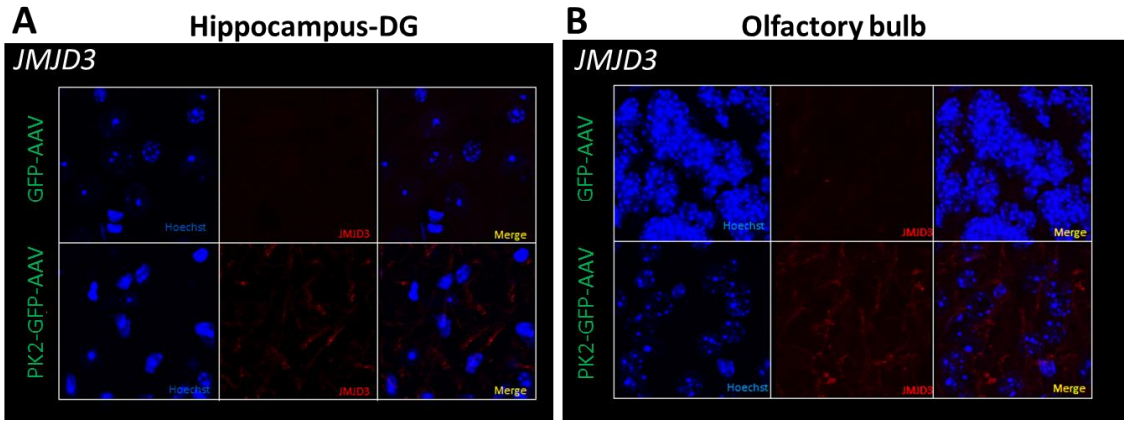

Figure S3. JMJD3 expression in neurogenic brain regions of MitoPark mice treated with GFP-AAV or PK2-GFP-AAV. An increase in JMJD3 expression is seen in the dentate gyrus of the hippocampus (A) and olfactory bulb (B) of PK2-GFP-AAV mice when compared to GFP-AAV treated MitoPark mice.

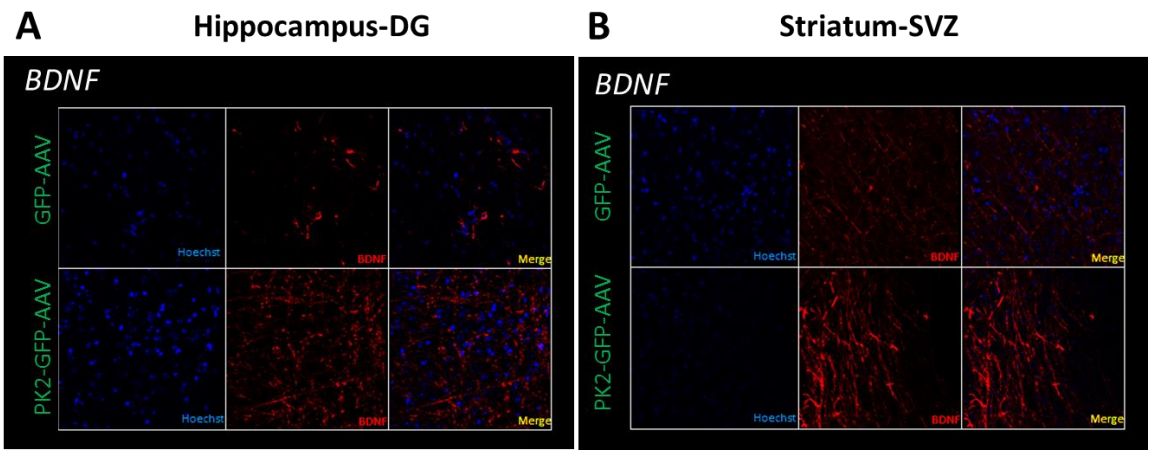

Figure S4. BDNF expression in neurogenic brain regions of MitoPark mice treated with GFP-AAV or PK2-GFP-AAV. An increase in BDNF expression is seen in the dentate gyrus of the hippocampal formation (A) and subventricular zone (SVZ, B) of PK2-GFP-AAV mice when compared to GFP-AAV treated MitoPark mice.

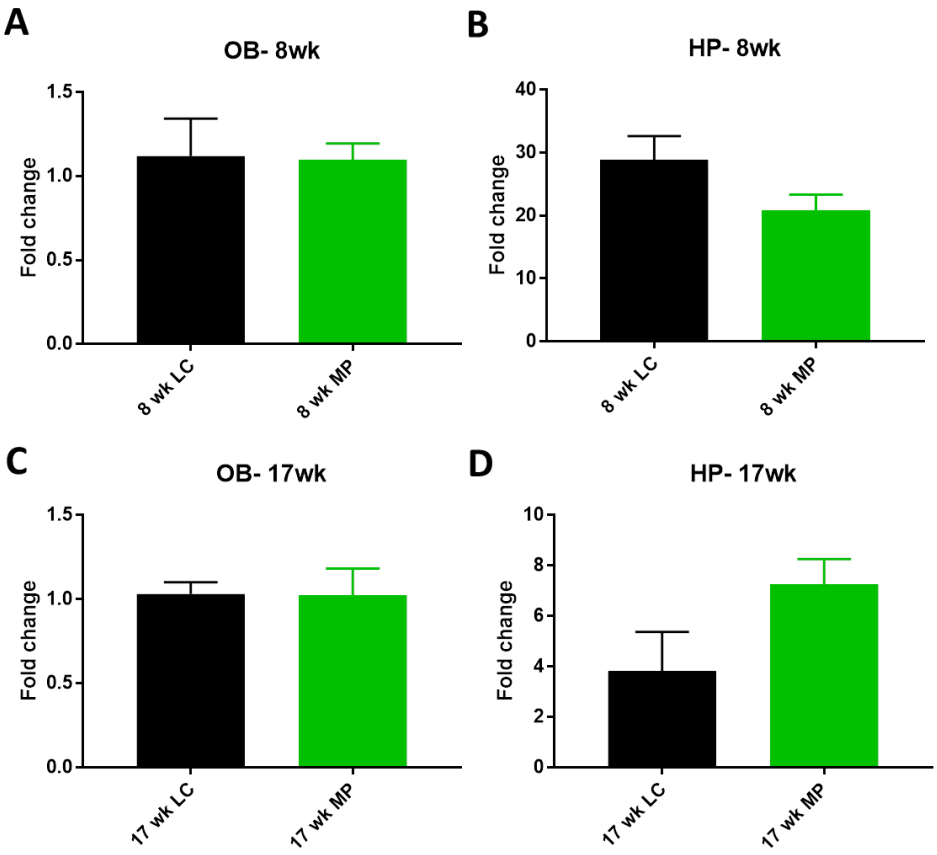

Figure S5. PK2 mRNA expression in neurogenic brain regions of MitoPark mice at various ages. No significant changes in PK2 mRNA expression were observed in MitoPark mice olfactory bulb (A, C) or hippocampus (B, D) tissues at eight (A, B) or 17 (C, D) weeks of age.

CHAPTER 6

GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

In this section, there is an overview of main results as described within the dissertation with an emphasis on future directions and impact of these conclusions. Key findings pertaining to the individual research chapters can be found in the "results" and "discussion" sections of the corresponding chapter.

The MitoPark Mouse as a Model of PD

Chapter 1 introduced the MitoPark mouse as a valuable transgenic PD animal model that recapitulates key features of human PD. Some of these known similarities include chronic, progressive neurodegeneration and motor deficits, protein aggregation, and adult-onset of disease related phenotypic changes (Ekstrand and Galter, 2009; Ekstrand et al., 2007). One major advantage over previously used models is that no toxin is administered, removing the possibility of toxicant-drug interactions during preclinical efficacy studies. Also, this results in less inter-animal variation because metabolism of the toxin or administration error is not a factor. Another beneficial feature is the slow, progressive nature of the motor deficits, which allows for studying the influence of environmental interactions or therapeutic efficacy when administered at various stages of disease progression. In particular, our data from Chapters 3 demonstrate the utility of young (8-12wk) MitoPark mice for gene-environment interaction studies due to the limited behavioral effects at this

age, allowing for toxicants to potentiate Parkinsonism symptoms and related pathophysiological processes. Whereas, Chapter 4 demonstrates the efficacy of a mitochondrially targeted antioxidant administered after motor deficits begin, analogous to when clinical diagnosis and treatment in human PD would typically begin.

MitoPark mice are L-DOPA responsive and even develop L-DOPA-induced dyskinesias (Galter et al., 2010; Gellhaar et al., 2015; Shan et al., 2015). Recent brain slice electrophysiology studies revealed a progressive decline in function of SN dopaminergic neurons from MitoPark mice as evidenced by decreased cell capacitance and increased input resistance (Branch et al., 2016). Dopamine signaling and expression of ion channel subunits was also affected in older MitoPark mice, revealing a potential new therapeutic target (Branch et al., 2016)**.** We have substantially contributed to the understanding of symptomology in this model by evaluating various nonmotor behavioral phenotypes from 8-24 wks of age in Chapter 2. Moreover, we have discovered that neuroinflammation, resulting from degenerating neurons, and decreased neurogenesis, resulting from dopamine deafferentation, are pathological features also present in the MitoPark model.

Although we have identified the advantages of using this model for neurotoxicity, behavioral, and neuroprotection studies, some disadvantages also exist. Some notable caveats of the MitoPark mouse model include the cost and size of breeding colonies needed, severity of the model making recovery particularly difficult, and the fact that TFAM is not directly known to contribute to PD pathogenesis (Alvarez et al., 2008; Cong et al., 2016; Grauer et al., 2014; Smith et al., 2014). For example, the modest recovery of function in MitoPark mice following Mito-apocynin treatment does not mirror the robust recovery seen in the toxin-based MPTP model (Dranka et al., 2014; Ghosh et al., 2016; Langley, 2017). However, the

severity of the MitoPark model could also be viewed as an advantage, given that many compounds which show success in animal studies end up failing in clinical trials. Although TFAM polymorphisms have not been directly associated with PD, mitochondrial dysfunction is certainly a common feature in both genetic and idiopathic PD pathology (Ekstrand and Galter, 2009; Ekstrand et al., 2007). A recent study has also shown that a single nucleotide polymorphism in the TFAM gene is associated with increased risk for Parkinson's disease dementia (PDD) (Gatt et al., 2013). Although this model looks into mitochondrial dysfunction in dopaminergic neurons, it would also be interesting to see the effect of TFAM knockdown on microglia by controlling Cre expression through the CX3CR1 promoter intead.

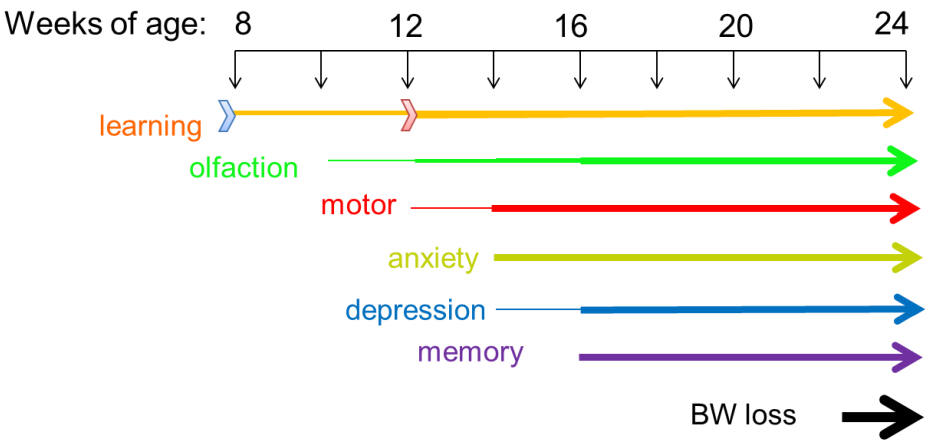

Figure 1. *Development of symptoms in MitoPark mice*

Nonmotor Symptoms are Evident in MitoPark Mice

The main objective of Chapter 2 was to determine which clinically relevant nonmotor symptoms exist in the MitoPark model and at what ages. Importantly, we have shown that MitoPark mice display olfactory deficits, cognitive dysfunction, depressive- and anxiolytic-

like behaviors. Furthermore, we identified key biochemical and neurochemical changes that correspond to the nonmotor symptoms observed.

Other groups have implicated that protein aggregation, dopamine deficiency, and receptor abnormalities potentially contribute to the development of olfactory dysfunction in PD patients and animal models (Doty, 2012; Ferrer et al., 2016; Ferrer et al., 2012; Hoglinger et al., 2015; Reichmann et al., 2016; Saito et al., 2016; Taylor et al., 2009). Herein, we revealed significant neurotransmitter changes in the olfactory bulb of aged MitoPark mice, but the role of protein aggregation and receptor function was not explored. In Chapter 3, 12 wk MitoPark mice did not show an increase in oligomeric proteins or a loss of dopamine. Later ages of MitoPark mice olfactory bulb should be used to determine effects on protein aggregation and olfactory receptors. Changes in other components involved in olfactory discrimination should also be explored including odor receptors in the nasal epithelium, individual glomerular and mitral layers of the olfactory bulb, piriform cortex, hippocampus, and thalamus (Hu et al., 2015; Li et al., 2015; Ravi et al., 2017). Researchers have used olfactory bulbectomy as a model of depression in rodents, and low olfaction correlated with a higher risk of apathy in PD patients (Hendriksen et al., 2015; Hong et al., 2015; Morales-Medina et al., 2017; Yuan and Slotnick, 2014). If the underlying olfactory pathology was able to be reversed by a therapeutic treatment, it would be particularly interesting to see if the depressed phenotype is also attenuated.

Because antidepressant treatment reserved immobility in the forced swim test, we can be confident that the depressive-like behavior observed is not confounded by the reduced locomotor activity. Moreover, CREB phosphorylation and BDNF protein expression, indicators of neurogenesis, did not change following forced swimming exposure in MitoPark

mice, unless MitoParks were administered intraperitoneal desipramine (an antidepressant which increases hippocampal neurogenesis). Thus, by increasing neurogenesis, the depressive-like immobility behavior was also attenuated.

Decreased hippocampal neurogenesis may also contribute to the cognitive deficits observed at later ages, such as those observed during the memory retention trial of Morris Water Maze (MWM). However, further studies are needed to elucidate the pathophysiology regulating deficits in the learning phase of MWM and Barnes maze, and deficits associated with impaired novel object recognition (Cong et al., 2016; Li et al., 2013b). On the other hand, increased CREB phosphorylation, BDNF expression, and lipid peroxidation in the striatum biochemically support depression, as they are seen in human depressed patients and animal models (De Vry et al., 2016; Han et al., 2016; Ma et al., 2016; Silva et al., 2016; Zhang et al., 2016a). The effect of L-DOPA therapy or dopamine agonists on depressive-like behavior in the MitoPark mice should also be examined, due to clinical studies suggesting that stimulation of dopamine D2-like receptors has antidepressant response (Blonder and Slevin, 2011; Gershon et al., 2007; Rana and Galani, 2014).

Sleep problems are a predictor of and a common problem associated with Parkinson's disease, but are cumbersome to thoroughly study in rodents (Comella, 2007; Faludi et al., 2015; Jauregui-Barrutia et al., 2010; Taylor et al., 2010). Within Chapter 2, we did not observe significant changes using the sleep latency test, a test mimicking tests used in humans for excessive daytime somnolence (Hunsley and Palmiter, 2004; Taylor et al., 2010) A recent publication did discover that MitoPark mice had an age-dependent decline in stability of their circadian rhythm, with increased fragmentation of diurnal and nocturnal activities (Fifel and Cooper, 2014). Following constant darkness or constant light conditions,

MitoPark mice were not able to maintain circadian rhythmicity (Fifel and Cooper, 2014). More advanced studies involving electroencephalography (EEG), polysomnography, and electromyography (EMG) could be performed to fully assess effects on REM sleep and other sleep problems, but typically require surgical implantation or intricate instrumentation (Hunsley and Palmiter, 2004; Mitchell et al., 2008; Tang et al., 2009). The sleep latency test as employed in Chapter 2 has been shown to correlate with EEG data (Hunsley and Palmiter, 2004; Mitchell et al., 2008). Dopamine depleted or hyperdopaminergic mice show differences in slow-wave and REM sleep that are regulated though the D2, but not D1 receptor (Dzirasa et al., 2006). Sleep is also understood to have effects on mood and gastrointestinal function, so links between these symptoms should be further explored in this model and others (Bishehsari et al., 2016; Borek et al., 2006; Taylor et al., 2010; Voigt et al., 2016).

Another prominent symptom of Parkinson's disease that has not yet been explored by others or within this dissertation is gastrointestinal problems such as constipation. Dopaminergic neurons in the enteric nervous system have been shown to be effected in MPTP and rotenone models of PD (Anderson et al., 2007; Murakami et al., 2015). Current studies in our laboratory to comprehensively characterize gastrointestinal function of MitoPark mice versus their littermate controls are currently underway (Ghaisas et al, 2017, unpublished).

Manganese Exacerbates and Accelerates Neurodegeneration

In Chapter 3, we revealed that low-dose Mn exposure can exacerbate the neurodegeneration and motor deficits in MitoPark mice at 12 wks of age. These key findings suggest that Mn exposure may accelerate disease progression in sensitive populations with mitochondrial

defects, caused by genetics or aging, by augmenting neurodegenerative processes. Improving our understanding of gene-environmental interactions in disease development could result in novel biomarkers, therapeutic strategies, and personalized medicine.

Studies have suggested that combining variability in the DAT gene with pesticide exposure interacts to increase odds-ratios in PD patients (Kelada et al. , 2006, Polito, Greco, 2016, Ritz et al. , 2009, Ritz et al. , 2016, Singh et al. , 2008).

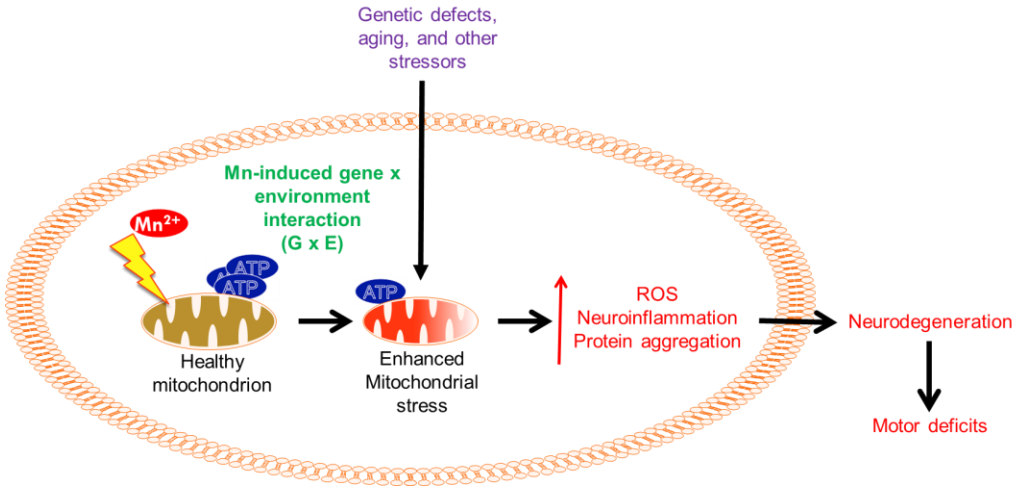

Figure 2. *Manganese-induced gene-environment interactions in MitoPark mice*

There are a number of confounding factors to consider when estimating environmental exposures to toxicants, making gene-environmental studies in humans challenging (Polito et al. , 2016, Thomas, 2010). Moreover, there has been controversy and opposing outcomes in studies involving Mn or welding fumes and the development of Parkinsonian syndromes (Guilarte and Gonzales, 2015, Nandipati and Litvan, 2016, Polito, Greco, 2016, Rentschler et al. , 2012, Sriram et al. , 2010). We believe we are the first to clearly show a novel synergistic effect of inherent mitochondrial dysfunction and metal exposure converging on oxidative stress, mitochondrial dysfunction, protein aggregation, and neuroinflammatory processes contributing to cell death in a PD animal model.

Future studies should direct attention to the underlying pathways in Mn's exacerbation of dopaminergic neurodegeneration at different ages, including developmental exposure of Mn, and more relevant exposure routes such as intranasal administration or inhalation. Furthermore, the potential interactions of the pathways involved and whether the effects seen are due to increased sensitivity to Mn or potentiation of mitochondrial dysfunction would provide valuable insight. We observed a significant decrease in ferroportin in the MitoPark mice, so the possibility of other Mn transporter expression changes contributing to Mn toxicity in the MitoPark model is another important avenue to explore in future experiments. Inductively coupled plasma mass spectroscopy (ICP-MS) analysis could help clarify if there is preferential accumulation in MitoPark mice versus controls or in particular brain tissues by allowing for quantification of Mn, Fe, and Zn levels.

Although we have shown anxiolytic and depressive-like behaviors in aged MitoPark mice and it is well known that Mn exerts neuropsychiatric effects, this sort of phenotyping was outside the scope of the current paper. However, such studies would be valuable and interesting if Mn also worsens anxiety and depression related behavior in elevated plus, tail suspension, and forced swim tests. Consistent with other reports (Casjens et al., 2017; Colin-Barenque et al., 2011; Lehallier et al., 2012), we showed olfactory deficits in our Mn-treated mice. However, we were not able to pinpoint the cause of the deficits in this model. No changes in protein aggregation or dopamine content were found in the olfactory bulbs of Mn-treated mice. In Chapter 2, we show that reduced neurogenesis begins at 16 wks in the SVZ and DG. It is possible that the migration of neural stem and progenitor cells is impaired at earlier ages in the olfactory bulb. Alternatively, Mn treatments could impair neurogenesis, although some studies suggest Mn actually increases neurogenesis (Fu et al., 2016). Instead,

olfactory epithelial cells and receptor function should be investigated as a mediator of olfactory dysfunction following Mn exposure (Chiu et al., 2015, Kurtenbach et al., 2013, Neuner, Filser, 2014, Postuma and Berg, 2016).

Mito-apocynin Inhibits Mitochondrial and NOX2-mediated Oxidant production, Thereby Offering Neuroprotection to MitoPark Mice

Conventional antioxidants have failed to produce significant effects in clinical trials for neurodegenerative disease, despite promising initial results in animal and cell culture studies (Bhatti et al., 2017; Jin et al., 2014; Reddy and Reddy, 2017). In Chapter 4, we have demonstrated that a mitochondrially-targeted antioxidant, Mito-apocynin, protected dopaminergic neurons and reduced oxidative and nitrative stress, glial cell activation and inflammatory reactions in the MitoPark mouse model. Several papers have demonstrated that the triphenylphosphonium cation (TPP+) targets compounds to mitochondria of intact cells (Dikalov, 2011; Murphy and Smith, 2000; Smith et al., 2003). Based on the reported structure-activity relationship, mito-apocynin is expected to rapidly

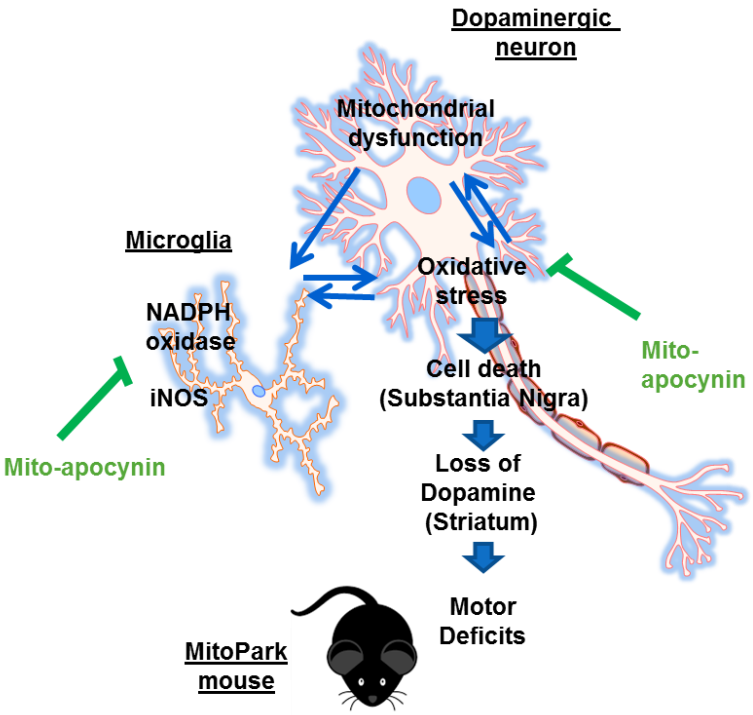

***Figure 3**. Mito-apocynin prevents neuroinflammation and oxidative stress in MitoPark mice.*

and efficiently accumulate in mitochondria. In addition, the protective effects on several mitochondrial parameters and cellular bioenergetic function also support mitochondria as an important target of mito-apocynin. Other antioxidants targeting mitochondria demonstrate neuroprotection by directly scavenging mitochondrial oxidants, thus reducing lipid peroxidation (Jin et al., 2014; Kumar and Singh, 2015). Apocynin has also been shown to have general antioxidant properties in addition to its role as an inhibitor/pro-inhibitor of NADPH oxidase (NOX) (Altenhofer et al., 2015; Heumuller et al., 2008). Importantly, we suggest that mito-apocynin is having a direct effect on mitochondrial function and radical scavenging properties.We further show that mito-apocynin improved mitochondrial morphology in neurons.

Since NOX2 is also considered a target (direct or indirect) of mito-apocynin (Dranka et al., 2014; Ghosh et al., 2016) we performed experiments to further identify the therapeutic target of mito-apocynin in microglia. Our data suggest that mito-apocynin can successfully prevent p47phox membrane recruitment, which is otherwise essential for NOX2 activation. These data clearly suggest that NOX2 inhibition is another major mechanism responsible for mito-apocynin's action. We propose that targeting mitochondrial and NOX2-mediated oxidant production by mito-apocynin breaks the vicious cycle of mitochondria-NOX ROS generation and thereby offers neuroprotection. To take things one step further, we also performed experiments to determine the specificity of mito-apocynin on NOX isoforms. Other groups have shown apocynin to be more specific to NOX2 than NOX4 (Serrander et al., 2007). Given that NOX4 localizes in the mitochondria of neurons (Case et al., 2013) and that mito-apocynin is mitochondrially targeted, we examined NOX4 levels; however, we found that NOX4 does not seem to be a target of mito-apocynin in our model systems.

Due to increased pro-inflammatory enzyme expression resulting in the production of nitric oxide and superoxide, peroxynitrite is formed, leading to nitration of tyrosine residues on proteins (Ghosh et al., 2016) . Mito-apocynin decreased the protein expression of iNOS, NOX2, and 3-NT *in vivo*. Based on the aforementioned data, we believe this combination of NOX2 inhibition and increased mitochondrial bioenergetic and antioxidant effects contributes to the neuroprotection observed in the MitoPark model.

NOXs play a direct role in dopaminergic neurodegeneration via ROS generation (Chen et al, 2016; Nayernia et al, 2014). However, their involvement in PD may also involve other contributing factors such as protein aggregation and mitochondrial dysfunction (Cristovao et al, 2012; Dikalov, 2011; Dranka et al, 2012). Such avenues should be explored in future experiments. Stressed neurons secrete factors that activate microglia, beginning a cycle of neuroinflammation and neuronal damage underlying neurodegenerative diseases deemed reactive microgliosis (Block and Hong, 2007; Levesque et al, 2010; Lull et al, 2010). Although neuroinflammation was not originally defined in the MitoPark model, we have shown here that reactive microgliosis is likely involved, implicating an important therapeutic potential for both mitochondrially targeted antioxidants and anti-inflammatory drugs in PD. Several papers have recently described morphological changes such as increased soma size and decreased branching complexity or de-ramification as correlates of microglial activation to a phagocytic phenotype (Healy et al., 2016; Hopperton et al., 2016; Morrison and Filosa, 2013; Orr et al., 2009). We have identified morphological changes indicative of microglia activation and increased IBA1+ microglia in the SN of MitoPark mice and also included CD68 immunostaining as another marker of microglia activation.

Although oxidative stress, neuroinflammation, and mitochondrial dysfunction are major contributors to cell death associated with PD, other mechanisms such as protein aggregation, ubiquitin-proteasome system dysfunction, mitochondrial fission and fusion, and inflammasome-dependent neuroinflammation are also important. These mechanisms may or may not be directly or indirectly affected by mito-apocynin. A lack of effect on these systems may also serve to explain why we observed a dramatic inhibition of aconitase activity (a marker for mitochondrial oxidative stress) by mito-apocynin treatment, but only modest effects on motor deficits, dopamine depletion and dopaminergic toxicity. These observations warrant future studies into the therapeutic role of mito-apocynin and the development of combination therapy modalities for PD.

Mito-apocynin may outperform other experimental drugs by being well tolerated and bioavailable in the brain when delivered orally. Its potent anti-neuroinflammatory and anti-oxidant effects were seen at a low dose in a chronic, progressive model of neurodegeneration when treatment began only after pathology was apparent. These properties strongly support potential clinical applications for Mito-apocynin as a viable neuroprotective and anti-neuroinflammatory drug for treating PD. While our data serves as an important proof-of-concept preclinical study in a progressive degenerative model of PD, we acknowledge that further GLP safety and efficacy assessment studies need to be done in rodent and primate models prior to clinical development of the drug.

Prokineticin-2 Promotes Proliferation and Differentiation of Neural Stem Cells

Currently available PD medicines only address symptoms and cannot prevent the progression of the neurodegenerative process. Approaches such as cell replacement or recruitment from neurogenic areas have gained popularity, despite difficulties with feasibility, ethical concerns, immunoresistance, and availability of tissue sources (Drouin-Ouellet, 2014; Hopperton et al., 2016). Difficulty with survival of grafts and getting the expanded cell cultures to differentiate into dopaminergic neurons has also been a huge obstacle as mentioned in Chapter 1 (Brundin et al., 2010; Deng et al., 2013; Espejo et al., 2000). In Chapter 5, we show that PK2 can promote proliferation and differentiation of neural stem cells, which could potentially lead to restoration of impaired neurogenesis or recruitment to damaged nigral tissue for replacing cells lost. PK2 is already known to be involved in normal SVZ-RMS-OB neurogenesis; however its function in the SGZ or during disease states remains unknown (Ng et al., 2005; Pitteloud et al., 2007; Zhang et al., 2007).

Previous work from our laboratory described a novel compensatory upregulation of PK2 in model and human PD tissues, and consequent protection from neuroinflammation and neurodegeneration in toxin-based animal models (Gordon et al., 2016). Further solidifying this finding, PK2 receptor antagonism by PKRA7 worsened disease progression (Gordon et al., 2016). In chapter 2, we had revealed that MitoPark mice show nonmotor symptoms of PD. Chapter 5 identified that a reduction in BrdU+ proliferative cells in the SVZ and DG may attribute to the nonmotor symptoms observed in the MitoPark mouse model. Stereotaxic injection of PK2-GFP AAV was able to attenuate olfactory and neuropsychiatric symptoms in aged MitoPark mice. Another current project from our laboratory identified a novel role of PK2 in glial cells through JMJD3 signaling via the PKR1 receptor (Neal, 2016). Because

neurogenesis is understood to be suppressed by glia activation (de Miranda et al., 2017; Fung et al., 2017; Xiong et al., 2016), more experiments should be done to elucidate the direct and indirect (via suppression of neuroinflammation) roles of PK2 in regards to altered neurogenesis in PD models.

Moreover, pharmacological inhibitors of key signaling pathways or CRISPR/Cas9 knockdowns should be used to tease out the exact mechanism of these downstream changes. Further BrdU co-labeling immunohistological studies at different time frames during the MitoPark mouse mice lifespan would help to identify the number, types, and migration to understand the complete range of impairments in neurogenesis in this model. Using the neurosphere assay, one could determine the differentiation potential of neural stem and progenitor cells of MitoPark mice in comparison to littermate controls. PCR array or sequencing studies could then identify key signaling regulators mediating the differences between mice. Overall, we identified a new role for PK2 overexpression by regulating adult neurogenesis through JMJD3, making it a promising new therapeutic target for gene therapy or for the development of novel CNS bioavailable PKR1 or PKR2 agonists.

NOMENCLATURE

4-HNE= 4-hydroxynonenal

6-OHDA= 6-hydroxydopamine

AAV = Adeno-associated virus

AD = Alzheimer's disease

ALP= alkaline phosphatase

ALS = Amyotrophic Lateral Sclerosis

AMS= AnyMaze software

ANOVA = Analysis of variance

α-syn = Alpha-synuclein

ATRA= all-trans retinoic acid

bHLH = basic Helix Loop Helix

BDNF = Brain derived neurotrophic factor

Bv8 = *Bombina Variegate* 8

CNS= central nervous system

CREB = cyclic AMP response element-binding protein

CRISPR= clustered regularly interspaced short palindromic repeats

DAB = 3,3’-Diaminobenzidine

DAT= dopamine transporter

DG= dentate gyrus

DOPAC= 3,4-dihydroxyphenylacetic acid

ECAR = Extracellular acidification rate

EPR= electron paramagnetic resonance

ERK = Extracellular signal-regulated kinase

FBS= fetal bovine serum

FST= forced swim test

GBA= glucocerebrosidase

GDNF = Glial cell-line derived neurotrophic factor

GFP = Green fluorescent protein

GI= gastrointestinal

GPCR = G-protein coupled receptor

GSM= grip strength meter

HPLC= high performance liquid chromatography

HVA= homovanillic acid

IBA1= ionized calcium-binding adapter molecule 1

ICC = Immunocytochemistry

IHC= immunohistochemistry

IL = Interleukin

ISU= Iowa State University

IS20 = Chemical Prokineticin receptor 1 agonist

iNOS= inducible nitric oxide synthase

HPLC= high performance liquid chromatography

JMJD3 = Jumonji-domain containing protein 3

KD= knockdown

L-DOPA= L-3,4-dihydroxyphenylalanine

LPS = Lipopolysaccharide

LRRK2= leucine-rich repeat kinase 2

MIT-1 = Mamba intestinal toxin 1

Mito-apo= Mito-apocynin

MTA= mitochondria-targeted antioxidants

MPP+ = 1-methyl-4-phenylpyridinium

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

MWM= Morris water maze

NO = Nitric Oxide

NSAID = Non-steroidal anti-inflammatory drug

NOX=NADPH oxidase

OB= olfactory bulb

OCR= oxygen consumption rate

OE = Overexpression

PINK1= PTEN-induced putative kinase 1

PK2 = Prokineticin-2

PKR1 = Prokineticin receptor 1

PKR2 = Prokineticin receptor 2

PMA= phorbol myristate acetate

PD= Parkinson's disease

qPCR= quantitative polymerase chain reaction

RMS= rostral migratory stream

ROS= reactive oxygen species

RNS = Reactive nitrogen species

SGZ= subgranular zone

SN= substantia nigra

STR= striatum

SVZ= subventricular zone

TFAM= mitochondrial transcription factor A

TH= tyrosine hydroxylase

TNFα= Tumor necrosis factor α

TST= tail suspension test

UPDRS = Unified Parkinson's disease rating scale

VTA = Ventral Tegmental Area

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