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Prokineticin 2 signaling: Genetic regulation and preclinical assessment in rodent models of Parkinsonism

Jie Luo
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Prokineticin 2 signaling: Genetic regulation and preclinical assessment in rodent models of Parkinsonism

by

Jie Luo

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

DOCTOR OF PHILOSOPHY

Major: Toxicology

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University
Ames, Iowa
2018

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DEDICATION

To God, my dear mother Leanne, and my girlfriend Haiyang
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ABSTRACT

Parkinson’s disease (PD) is the second most common neurodegenerative disorder after Alzheimer’s disease. The etiology of PD is currently not fully understood, but strong evidence has existed pointing to gene-environmental interactions that contribute to various pathogenic mechanisms of neurodegeneration: oxidative stress, neuroinflammation, mitochondrial dysfunction, and epigenetic modulation. While cell signaling mechanisms underlying neurotoxic injury have been actively studied in recent years, the signaling molecules contributing to compensatory survival signaling are largely unknown. Recently, we reported that the secreted neuropeptide prokineticin-2 (PK2) is upregulated during early stages of neurotoxic stress induced by MPP+, and plays a major compensatory protective function in nigral dopaminergic neurons in vitro and in vivo. Thus, the goal of my thesis work was to determine the transcriptional regulation and translational relevance of PK2 in cell culture and animal models of PD.

In Chapter 2, we studied the transcriptional and molecular regulatory mechanisms of Parkinsonian toxicants MPP+ and manganese (Mn)-induced PK2 upregulation. In a set of experiments, we performed in silico analysis of the PK2 promoter and revealed the presence of early growth response-1 (EGR1), hypoxia inducible factor 1 (HIF1α), transcription factor E2F, and nuclear respiratory factor-1 (NRF1) putative binding sites. Importantly, we observed that MPP+ and Mn exposure increased HIF1α and EGR1 levels at early stages of neurotoxicity. Since overexpression of HIF1α or EGR1 upregulated PK2 expression, these studies suggest that Mn regulates PK2 expression via EGR1 or HIF1α-dependent pathway. A toxicologically relevant dose of Mn exposure by an oral route significantly upregulated global PK2 levels in the brain, especially in the substantia nigra.
with concomitant increases in HIF1α transcription factor. Taken together, the results in this chapter suggest that dopaminergic neurotoxicants upregulates PK2 levels to counter the early neurotoxic stress, and that Mn-induced upregulation of PK2 expression is transcriptionally regulated by EGR1, HIF1α and NRF1.

Building on the findings in chapter 2, the focus of Chapter 3 is the role of PK2 in Mn-induced Parkinsonism because the cellular mechanism by which Mn impairs the dopaminergic neurotransmitter system remains unclear. Here we found that the dopaminergic neurotoxicant Mn rapidly induce PK2 during the early stages of Mn neurotoxic stress in an N27 dopaminergic cell model. To better understand the functional role of PK2 upregulation, we created stable PK2-expressing dopaminergic cells by delivering PK2 myc-tagged cDNA into mouse dopaminergic MN9D cells. Interestingly, PK2-overexpressing cells exposed to Mn showed significant protection against neurotoxicity relative to vector control cells, suggesting a neuroprotective role for PK2 in dopaminergic neurons. The protective effect was both dose- and time-dependent. Furthermore, the PK2 receptor blocker PC7 attenuated the PK2-induced neuroprotective effects in PK2-overexpressing cells. Overexpressing PK2 protected against manganese-induced apoptosis as measured by Annexin 5 and caspase-3 activation. We also found that mitochondrial integrity was well maintained in PK2-overexpressing cells relative to vector cells following exposure to Mn. Additional results also showed that key proteins involved in mitochondrial functions, including BCL2, PGC1-α and TFAM levels, were preserved in PK2-overexpressing cells during neurotoxic stress. Collectively, our results suggest that neurotoxic insults upregulate PK2 in dopaminergic neurons to protect against the early stages of neurotoxicity. Finally, observed the effect of Mn treatment on PK2 expression
using a GENSAT PK2 GFP transgenic mouse model. During early exposure to Mn, PK2 levels were significantly upregulated in the SN while slightly decreasing in striatum. Prolonged exposure to Mn (30 mg/kg for 30 days) significantly upregulated PK2 levels in the brain, especially in the substantia nigra. Interestingly, in the striatum, where Mn-induced cell death mainly occurs, decreased PK2 levels were noted. Significant amount of methylation of the PK2 promoter region is also observed in the striatum. Combined with cell culture studies, the differential regulation of PK2 in the striatum and substantia nigra might suggest a possible neuroprotective role of PK2 in the SN during early exposure to Mn. Taken together, the results in this chapter suggest that Mn upregulates PK2 levels to counter early neurotoxic stress \textit{in vitro} and \textit{in vivo}.

Despite a wealth of preclinical studies establishing neuroprotective and neurorestorative properties of glial cell-line derived neurotrophic factor (GDNF) in animal models of PD, a number of phase II clinical trials utilizing direct intracranial injection of GDNF protein and AAV-mediated Gdnf gene transfer did not achieve efficacy that was hoped for. Setbacks from recent clinical trials might prompt a rethinking of the strategy which focused on ectopic expression of GDNF targeted towards neurons. Devising strategies to elevate GDNF expression by means other than genetic manipulation is the current challenge. The pharmacologically modulated signaling pathways that are in crosstalk with GDNF or could induce its endogenous upregulation represent opportunities to fully harness the clinical benefits of GDNF, without the side effects associated with current methods for GDNF delivery. In Chapter 4, we show that GDNF has significant crosstalk with prokineticin signaling in astrocytes. A small molecule, IS20, could activate prokineticin signaling to induce secretion and expression of GDNF from in cultured
astrocytes via activation of prokineticin receptor 1 (PKR1) preferentially expressed by astrocytes. Further, non-invasive administration of the blood-brain-barrier-permeable lipophilic IS20 through intranasal delivery could pharmacologically modulate GDNF levels in the nigrostriatal system of C57B/6 mice. Importantly, IS20 treatment yielded significant neuroprotective and neurorestorative effects in the MPTP and MitoPark mouse models of PD. Our results indicate that the full clinical benefits of GDNF could be leveraged by pharmacological modulation using IS20.

In summary, we showed that PK2 could protect against classic Parkinsonian toxicants MPP⁺ and Mn induced neurotoxicity in MN9D mouse dopaminergic neuronal cells. We characterized the transcriptional regulation of PK2 by analyzing the PK2 promoter, and the results revealed that transcriptional factors HIF1α, EGR1, and NRF1 contribute to basal and neurotoxicity-induced PK2 expression. While studying the PK2 neuroprotective mechanisms, we found a fundamental relationship between PK2 and GDNF signaling pathways in astrocytes. Activation of PK2 signaling upregulates GDNF levels in vitro and in vivo. The protective role and therapeutic potential of the PK2-PKR1-GDNF signaling axis was further confirmed using a PKR1 agonist, IS20, in a MPTP mouse model of neurodegeneration as well as in the MitoPark genetic mouse model of PD. Collectively, we show that PK2 can be transcriptionally induced by multiple pro-survival factors and that PK2 signaling activation is a protective compensatory response to neurodegeneration. Pharmacological modulation of PK2 could induce GDNF upregulation and offer neuroprotective effects in multiple mouse models of PD. Thus, PK2 signaling represents a therapeutic target with great potential for PD treatment.
CHAPTER 1. GENERAL INTRODUCTION

Dissertation Layout and Organization

This dissertation is written in the alternative format which consists of manuscripts that are in preparation for submission to peer reviewed journals. The dissertation contains the General Introduction, followed by three research manuscripts as individual chapters, and ends with the Conclusion and Future Directions chapter. The references are listed at the end of the dissertation, except for each of the three manuscript chapters, which are followed immediately by works that are cited within the manuscript chapter. The General Introduction (Chapter 1) provides a concise review of current knowledge and hypotheses for the etiology of Parkinson’s disease, and the role of genetics and environmental exposures, especially heavy metal exposures. Recent and current clinical trials for experimental PD therapies are discussed, as well as their shortcomings. The role of Prokineticin-2 (PK2) in PD is discussed, in relevance to studies presented here in the following three research manuscripts. The first research manuscript (Chapter 2) is titled: Transcriptional Regulation Of Prokineticin-2 Promoter By Egr1, Hif1α, And Nrf1 in Cell Cultures of Dopaminergic Neurodegeneration. The second research manuscript (Chapter 3) is titled: Prokineticin 2 Protects Against Manganese-Induced Neurotoxicity by Inducing Mitochondrial Biogenesis in Dopaminergic Cells. The third research manuscript (Chapter 4) is titled: Pharmacological Modulation of Astrocyte-Derived GDNF by Prokineticin 2 Receptor Agonist IS20: Preclinical Assessments Using MPTP And MitoPark Rodent Models of Parkinson’s Disease. All of the above manuscripts are in preparation for submission to peer-reviewed journals.
This dissertation contains the experimental results obtained by the author during his Ph.D. study, under the supervision of his major professor Dr. Anumantha G. Kanthasamy at Iowa State University, Department of Biomedical Sciences.

**Introduction**

Parkinson’s disease (PD) is a complex neurological disorder. It is the most common neurodegenerative disorder after Alzheimer’s disease, affecting about 1.5 million people in the United States and over 10 million worldwide. With an aging world population, it is projected to increase by more than 50% by 2030 (Dorsey et al., 2007). It will cost a national economic burden in the United States exceeding $13 billion in by 2030, and $18 billion by 2050 (Kowal, Dall, Chakrabarti, Storm, & Jain, 2013).

In 1817, James Parkinson described a disease which is characterized today by akinesia, bradykinesia, rigidity, postural instability and fatigue (Barzilai & Melamed, 2003; Jarraya et al., 2009; Nassif & Pereira, 2018), a set of conditions with the earliest reference dating back to 600 B.C. in the ancient Indian medical literature (Manyam, 1990; Ovallath & Deepa, 2013). Current understanding of PD indicates that the early death of dopaminergic neurons in the ventrolateral substantia nigra pars compacta (SNpc), and the resulting severe dopamine depletion in the dorsal caudate putamen sit at the core of the disease. Postmortem analysis of PD patient brains has found another hallmark of PD: the accumulation of Lewy bodies in the neuron bodies and Lewy neurites in neuron processes, which comprised of mainly alpha-synuclein aggregates. The role of Lewy bodies and PD pathogenesis still stands unclear (Recasens et al., 2014; Wakabayashi et al., 2012).

PD diagnosis by clinicians is aided by medical imagining using PET scans, which could assess the functional integrity of the nigrostriatal dopaminergic system (Niccolini, Su, &
Politis, 2014; Poewe, 1993). However, PD is diagnosed incorrectly in about 25% of patients, in part due to a lack of laboratory diagnostic tests for definitive diagnosis (Kalia & Lang, 2015; Tolosa, Wenning, & Poewe, 2006). The definitive diagnosis only comes after post-mortem histopathological examination of degenerated dopaminergic neurons in the brain of patients. Motor deficits occur when an estimated 60% of the dopaminergic neurons in the SNpc are lost with 80% reduction in striatal dopamine levels, attesting to the brain’s immense compensatory capacity for reduced dopamine production during early stage of the disease, while underscoring a need for early diagnosis independent of motor symptoms. Non-motor symptoms associated with PD have been increasing recognized and used in the differential diagnosis of PD from other similar conditions during the last decade (Richard L. Doty, 2012; Rodríguez-Violante, Zerón-Martínez, Cervantes-Arriaga, & Corona, 2017). Because of prevalence of non-motor symptoms (over 90%) in PD patients, non-motor symptoms can be taken into consideration during differential diagnosis, together with motor symptoms and medical imagine data, to lower the rate of misdiagnosis. Because non-motor dysfunctions appear before motor dysfunctions, the progressive loss of dopaminergic neurons has presumably already begun, during the manifestation of non-motor symptoms, with its effects unseen until various compensatory mechanisms are overwhelmed. Therefore, the prodromal period preceding massive dopaminergic cell death represents a critical window of opportunity for a potential therapeutic intervention to alter the course of disease progression (Siderowf & Lang, 2012).

The first mutations in SNCA (PARK1) were found to be responsible for PD in 1997 (M H Polymeropoulos et al., 1997; Mihael H. Polymeropoulos et al., 1996). Since then, with help from large scale genome-wide association studies (GWAS) enabled by advances in sequencing technology, a total of 28 mutations in various genomic loci have since been linked
to increased risks of PD (Klein et al., 2012; Simón-Sánchez et al., 2009). However, PD patients who have mutations in these genes comprise of only roughly 5% of clinical cases (Pankratz et al., 2012; Shulman, De Jager, & Feany, 2011), indicating that rather than being purely hereditary, the etiology of PD is multifactorial, involving an interplay of PD-susceptibility genes that are modified or influenced by environmental factors, leading to a cascade of events which result in PD pathology.

A meta-analysis had found 11 environmental factors that have positive association with PD, and the top risk factors are pesticide exposure, well-water drinking, beta-blocker use, prior head injury, agricultural occupation, and rural living (Foubert-Samier et al., 2012). Exposure to pesticides such as rotenone, paraquat, dieldrin, organophosphates and Mn (in the form of pesticide Maneb) are implicated in development of PD (A. G. Kanthasamy, Kitazawa, Yang, Anantharam, & Kanthasamy, 2008; Roede & Miller, 2014; Semchuk, Love, & Lee, 1992; Singh et al., 2018; Tanner et al., 2011). In addition, aerosolized metals exposure has been reported as an environmental risk factor for PD (Brown, Lockwood, & Sonawane, 2005). In particular, exposure to manganese in an occupational setting could increase risks for developing Parkinsonian symptoms by 3 - 10 fold (Gorell et al., 1999). Therefore, studying both the environmental and genetic factors of PD could provide insight into possible points of failure within the cell caused by mutations in the PARK genes, that can be exacerbated by environmental factors, leading to neurodegeneration.

Collectively, the consensus might be that PD is a multifactorial disease with contributions from genetic and environmental sources, which converge on the vulnerabilities of the dopaminergic neurons in the SNpc. Looking beyond the risk factors of the disease, some of which are inherently unavoidable, several common themes appearing during the
neurodegenerative process have emerged. Oxidative stress, neuroinflammation, and mitochondrial dysfunction are seen from in vitro, in vivo studies, and post mortem analyses of PD patient brains. Until recently, these processes that accompany nigral dopaminergic degeneration was presumed to be consequences of cell death. However, over the past decades, evidence had pointed to their active participation in neurodegeneration and had implicated them in PD pathogenesis and disease progression.

The mainstay of PD symptomatic management remains to be therapies that increase dopamine concentrations or receptor activity in the caudate putamen. L-DOPA, the precursor for dopamine, was discovered by Arvid Carlsson in the 1950s to have effects on Parkinsonian animals, experiments for which the Nobel Prize in Physiology or Medicine was awarded in 2000. Decades later, L-DOPA remains today the strongest therapy against disease symptoms. However, common therapies such as L-DOPA, dopamine agonists, monoamine type B inhibitors, induce side effects such as dyskinesia with long-term treatments. Without disease-modifying treatments, current therapies could manage motor symptoms but do not treat non-motor symptoms, and do little to slow disease progression.

One of the most promising candidates for neuroprotection and neuro-restoration of SNpc dopaminergic neurons are members of the glial-cell line derived neurotrophic factor (GDNF) family, consisting of GDNF, artemin, persephin, and neurturin. Since the discovery of GDNF in 1992 (O’Malley, Sieber, Black, & Dreyfus, 1992), extensive evaluation in cell culture, rodent, and primate models of PD had demonstrated its exceptional neuroprotective effects on the dopaminergic system (Bilang-Bleuel et al., 1997; Björklund, Rosenblad, Winkler, & Kirik, 1997; J. H. Kordower et al., 2000; L. F. Lin, Doherty, Lile, Bektesh, & Collins, 1993; Tseng, Baetge, Zurn, & Aebischer, 1997). Nerve injury induces upregulation of
GDNF (Araujo & Hilt, 1997), and GDNF seem to be upregulated in post-mortem brains of PD patients in some studies (Backman et al., 2006). Therefore, GDNF could be a protective compensatory mechanism against neurodegeneration. Several landmark GDNF clinical trials had initially showed relative safety and efficacy in open-label studies, but ultimately, GDNF delivery using currently employed adeno-associated virus (AAV) showed no efficacy in phase II clinical trial (Blits & Petry, 2016; Remy, 2014; Tenenbaum & Humbert-Claude, 2017). Hence, the greatest challenge in meeting unmet medical need for PD remains to be the development of disease course-modifying therapies that could slow or stop the progression of the disease (Kalia & Lang, 2015).

We had recently discovered that a neuropeptide, prokineticin-2, is upregulated in response to neurotoxic stress. We found that its upregulation is a protective, compensatory response during neurodegeneration in animal models of PD. We had also found that it protects against neurodegeneration by activating pro-survival ERK1/2, AKT pathways, countering pro-apoptotic signals, and bolstering mitochondrial biogenesis (Gordon et al., 2016). We recently have also found that PK2 reduces neuroinflammation by activating pro-survival astrocytic phenotype. Yet, little is known about the regulation of PK2 during neurotoxic stress in dopaminergic neurons, or the relationship of PK2 with other neurotrophic factors co-regulated with it during neurotoxic stress. The focus of the following chapters will center around PK2’s role during neurotoxic stress—PK2’s transcriptional regulation, PK2’s role in manganese model of Parkinsonism, and the role of PK2 in regulation of GDNF expression.
Background and Literature Review I

The goal of this section is to summarize the pathophysiology of Parkinson’s disease and current understanding of its etiology through discussion of environmental risk factors as well as genetic risk factors for Parkinson’s disease. The proposed mechanisms and hypotheses for disease progression will be presented. Parkinson’s disease animal models relevant to the research presented in this work will also be discussed.

Parkinson’s Disease

Parkinson’s disease (PD) is the second most common neurodegenerative diseases behind Alzheimer’s disease. The mean onset for PD is 60 years of age, and it affects 10 million people worldwide, or 4-5% of people over 85, for whom mortality rate is 2-3 times that of the general population (de Lau et al., 2006; Lang & Espay, 2018). Its prevalence and incidence nearly exponentially increase by age, and peak after 80 years of age (Callesen, Scheel-Krüger, Kringelbach, & Møller, 2013; Driver, Logroscino, Gaziano, & Kurth, 2009). Each year, 50,000 new cases of PD are diagnosed in the US alone, and the more than 1 million PD patients in the US exact a yearly national economic burden exceeding $14.4 billion in 2010 and is projected to exceed $ 18 billion by 2050 (Kowal et al., 2013). Due to relative complex etiologies of neurodegenerative diseases such as PD, they remain largely incurable, and are projected by the World Health Organization to overtake cancer as a leading cause of death by 2050 (Menken, Munsat, & Toole, 2000). Due to its progressively debilitating nature, PD is one of the most feared diseases of mankind (Fan et al., 2015).

In his famous monograph "An Essay on the Shaking Palsy" in 1817, James Parkinson described a condition characterized by “Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk
forwards...” (Parkinson et al., 2002), a condition with the earliest reference dating back to 600 B.C. in the ancient Indian medical literature by the name of Kampavata (Manyam, 1990; Ovallath & Deepa, 2013). Today, PD is characterized clinically by akinesia, bradykinesia, rigidity, postural instability and fatigue (Barzilai & Melamed, 2003; Jarraya et al., 2009; Nassif & Pereira, 2018). The degree and severity of the motor disability is measured clinically by the Unified Parkinson’s Disease Rating Scale (UPDRS) that measure motor functions of activities of daily living (Fahn & Elton, 1987). The pathophysiology of PD is characterized by the death of dopaminergic neurons in the SNpc resulting in dopamine deficiency in the caudate putamen and leading to motor symptoms. Post-mortem analysis of PD patient brains also revealed accumulation of Lewy bodies in dopaminergic neurons, consist largely of insoluble alpha synuclein, which seem to accompany dying neurons and is therefore the second hallmark of PD. Although is found to be protective during early stages of PD (Harischandra, Jin, Anantharam, Kanthasamy, & Kanthasamy, 2015), during later stage of PD, the misfolding of α-synuclein and accumulation of Lewy bodies are thought to be an essential mechanism causing the lesions seen in PD and dementia with Lewy body (Chartier & Duyckaerts, 2018). PD is also associated with numerous non-motor symptoms, include: hyposmia, taste perception deficit, psychosis, depression constipation, sleep disturbances, and sexual dysfunctions, all of which further reduce quality of life for PD patients (Cecchini et al., 2014; Richard L. Doty, 2017; Friedman, 2013; Martinez-Martin, 2011; Masala et al., 2018; Pfeiffer, 2016; Rossi, Merello, & Perez-Lloret, 2015; Santos-García & De La Fuente-Fernández, 2013; Thobois, Prange, Sgambato-Faure, Tremblay, & Broussolle, 2017; Varanda et al., 2016; Videnovic & Golombek, 2013) Interestingly, these non-motor symptoms generally do not correlate with UPDRS scores or disease duration, and some symptoms, such as olfactory function, as
measured by University of Pennsylvania Smell Identification Test (UPSIT), is correlated with age (Haehner, Hummel, & Reichmann, 2009). Together with other symptoms such as sleep disturbances, these non-motor symptoms could precede the development of motor symptoms by a decade or more (Postuma et al., 2012). Although the etiology of the disease is still largely not understood, our understanding of the molecular mechanisms behind PD has greatly advanced in the last two decades.

Genetic Risk Factors in PD Pathogenesis

Until two decades ago, PD was not considered to have a genetic basis (Billingsley, Bandres-Ciga, Saez-Atienzar, & Singleton, 2018). Advances in molecular genetics and sequencing technology had enabled the discovery of underlying causes of PD for several families with increased PD occurrence. SNCA mutation discovered in 1997 was the first mutation found to cause monogenic PD (M H Polymeropoulos et al., 1997), where a single mutation is sufficient to cause the disease. LRRK2 mutation was discovered in 2004 (Zimprich et al., 2004), the most common cause of monogenic PD. To date, six genes, SNCA (PARK1), LRRK2 (PARK8), Parkin (PARK2), PINK1 (PARK6), DJ-1 (PARK7), and ATP13A2 (PARK9) are found to cause monogenic PD. Mutations in these genes comprise of 30% of familial PD cases, and 3–5% of sporadic PD cases. Beyond mutations in genes that cause monogenic PD, mutations in other genes have been found to contribute as risk factor for PD. PRKN, PINK1, and DJ-1 mutations are associated with early-onset PD, and altogether, 28 distinct chromosomal regions have been found to be associated with PD (Klein et al., 2012). Yet, mutations in these genes accounts for only 10% of all cases of PD, while most cases of PD remain idiopathic. It had therefore come to light that PD is most likely multifactorial,
resulting from gene-environment interactions that act on mutated susceptibility alleles present in the patient, to impact the aging brain.

**Environmental Risk Factors and Role of Mn Exposure in development of PD and PD-Like Pathologies**

A large-scale meta-analysis analyzed 30 potential risk factors for developing PD (Noyce et al., 2012). Interestingly, tobacco smoking was negatively associated with PD, but the association is confounded by the fact that early-stage PD patients experienced decreased responsiveness to nicotine and therefore quit tobacco use more often (Shahi & Moochhala, 1991). Although several other studies had found protective effects of nicotine in tobacco smoke and suggested its role in PD therapy (Barreto, Iarkov, & Moran, 2015; Ma, Liu, Neumann, & Gao, 2017), a history of smoking increased the risk of dementia in PD by almost two fold (Yaqian Xu, Yang, & Shang, 2016) and the nature of its effect on PD remain controversial (Ascherio & Schwarzchild, 2016; Shahi & Moochhala, 1991). The meta-analysis had found 11 environmental factors considered as risk factors for PD. Among them, the top risk factors are pesticide exposure, well-water drinking, beta-blocker use, prior head injury, agricultural occupation, and rural living (Foubert-Samier et al., 2012). Exposure to pesticides such as rotenone, paraquat, dieldrin, organophosphates and Mn (in the form of pesticide Maneb) are implicated in development of PD (A. G. Kanthasamy et al., 2008; Roede & Miller, 2014; Semchuk et al., 1992; Singh et al., 2018; Tanner et al., 2011). Additionally, exposure to Mn in an occupational setting could increase risks for developing Parkinsonian symptoms by 3 - 10 fold (Gorell et al., 1999). Longitudinal cohort study of 886 welding-exposed workers had found yearly changes in UPDRS scores dependent on dose of Mn exposed, which was especially severe in flux core arc welders who work in a confined space (Racette et al., 2017).
Farmers exposed to Mn-containing pesticides Maneb and Mancozeb also showed adverse neurological effects (Thrash, Uthayathas, Karuppagounder, Suppiramaniam, & Dhanasekaran, 2007).

**Mn exposure, target organ, clinical features, pathophysiology**

Mn is a trace element found in all life on earth. It is an essential cofactor needed for such fundamental cellular processes as metabolism of fats and carbohydrates, regulation of blood sugar, and calcium absorption (Erikson, Syversen, Aschner, & Aschner, 2005). Mn is a cofactor in the reactive catalytic centers of essential enzymes manganese catalase and MnSOD, both of which convert and reduce oxidants (Species, Finkel, & Species, 2001). Mn exists in various chemical forms including several oxidation states (Mn$^{2+}$, Mn$^{3+}$, Mn$^{4+}$) (Rask, Miner, & Buseck, 1987; Reaney & Smith, 2005), a property which enabled its wide industrial uses. Current safety measures may not adequately protect welders from aerosolized metals, and consequently, risks of overexposure to Mn for workers such as welders and miners in occupational settings are increased, usually via dermal absorption and inhalation routes. Excessive absorbed Mn can accumulate in the brain, a major organ of Mn toxicity during overexposure, where it preferentially concentrate in globus pallidus and striatum in monkeys (Dastur DK, Manghani DK, 1971; Fujii, 1975) and humans (Aschner, 2006; Aschner, Guilarte, Schneider, & Zheng, 2007; Sarkar et al., 2018; Wooten, Aweda, Lewis, Gross, & Lapi, 2017; Yokel, 2009), to cause cell death in the basal ganglia and disruption of the nigrostriatal pathway. Disruption of several neurotransmitter systems, particularly the dopaminergic system, results in pathology that manifests itself in a set of extrapyramidal symptoms similar to Parkinson’s disease, called manganism: postural instability, bradykinesia, micrographia, and
a characteristic cock-walk caused by dystonia of the legs (Cersosimo & Koller, 2006; C. W. Olanow, 2004; Perl & Olanow, 2007). Due to these similarities, Mn exposure has been used as a mouse model for PD since 1973 (Villalobos et al., 2009). Studies assessing Mn toxicity are listed in Table 1. Furthermore, mitochondrial dysfunction and oxidative stress processes occurring in PD are strikingly similar to the neurodegenerative processes occurring also in manganism, suggesting that these dysregulated processes could be common in both manganism and PD.

Table 1. Studies showing a wide range of doses used for Mn treatment in rodents.

<table>
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<th>Dose, Route of Administration and Duration</th>
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<td>Oral, 5.6mg/kg/day, 30 days</td>
<td>Decreased performance in maze test</td>
<td>Shukakidze et al., 2003</td>
</tr>
<tr>
<td>White Rats</td>
<td>Oral, 6.5mg/kg/day, 30 days</td>
<td>Decreased activity in open field test</td>
<td>Vezer et al., 2005, 2007</td>
</tr>
<tr>
<td>Sprague-Dawley Rats</td>
<td>Oral, 328mg/kg/day, 21 days</td>
<td>Increased acoustic startle response</td>
<td>Golub et al., 2005</td>
</tr>
<tr>
<td>C57B/6 mice</td>
<td>Intranasal, 30mg/kg/day, 21 days</td>
<td>Decreased activity in open field test</td>
<td>Johnson et al., 2018</td>
</tr>
<tr>
<td>C57B/6 mice</td>
<td>Oral, 30mg/kg/day/21 days</td>
<td>Decreased activity in open field</td>
<td>D.-S. Kim et al., 2017</td>
</tr>
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</table>
Mn mechanisms of toxicity: transport, mitochondrial accumulation, induction of epigenetic changes

In the body, Mn can be transported into the cell by a few metal transporters: divalent metal transporter 1 (DMT1), bicarbonate ion symporters ZIP8 and ZIP14, transferrin receptor (TFR), solute carrier-39 (SLC39) family of zinc transporters, ATP13A2, and magnesium transporter HIP14. Among these, DMT1 and TFR are most studied in their function to transport Mn. In the cell, Mn is sequestered to the mitochondria by Ca\(^{2+}\) uniporter (Gunter, Gavin, Aschner, & Gunter, 2006), and gradually increase in concentration due to extremely slow efflux (Gavin, Gunter, & Gunter, 1990; Martinez-Finley, Gavin, Aschner, & Gunter, 2013). Mn treatment in cells has been shown to increase oxidative stress, and some studies had shown that Mn could inhibit mitochondrial efflux of Ca\(^{2+}\), thus increasing Ca\(^{2+}\) concentration and leading to overproduction of ROS (Tjalkens, Zoran, Mohl, & Barhoumi, 2006).

In dopaminergic neuronal cells, Mn could cause caspase-3-dependent PKC\(\delta\) cleavage of an active PKC\(\delta\) product (41 kDa) capable of translocating into the nucleus for expression of proapoptotic genes (Masashi Kitazawa et al., 2005; Latchoumycandane et al., 2005; D. Zhang, Kanthasamy, Anantharam, & Kanthasamy, 2011). Meanwhile, PKC\(\delta\) also negatively regulates tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, by enhancing protein phosphatase-2A activity in dopaminergic neurons (D. Zhang et al., 2011). Since Mn preferentially accumulates in the basal ganglia, it could profoundly affect dopamine release from dopaminergic neuronal terminals in the striatum (Guilarte, 2010). Furthermore, Mn exposure is capable of inhibiting the acetylation of core histones in SH-SY5Y cell models of PD (Guo et al., 2018) and inducing epigenetic changes in several PD-associated PARK genes (Tarale et al., 2017). Maternal developmental Mn exposure in mice could affect neurogenesis
of newborn mice and increased promoter hypermethylation and transcript down-regulation of large number of genes even through postnatal day 77 (L. Wang et al., 2013).

Currently, Mn overexposure is treated with chelation therapy for immediate sequestration of remaining Mn in body fluids, most commonly with edetate calcium disodium (EDTA), and more recently, with para-aminosalicylic acid (PAS), for the primary goal of removing the patient from further exposure (J. Lee, 2000). The extrapyramidal symptoms of manganism patients initially respond to L-DOPA treatment after short-term observation (Mena, Court, Fuenzalida, Papavasiliou, & Cotzias, 1970), but failed to show significant benefit from L-DOPA treatment in patients with chronic manganese poisoning. Furthermore, no treatment is available for the neurorestoration of degenerated neurons affected by Mn overexposure. Since cellular mechanisms of toxicity overlaps with PD, the common pathways of neuronal degeneration are similar between manganism and PD, the differences of manganism vs PD arises out of preferential accumulation of Mn in basal ganglia. It is therefore speculated that protective strategies against neurodegeneration caused by Mn overexposure could also have similar protective effects against neurodegeneration in PD.

**Induction of Neuroinflammation from Exposure to Environmental Neurotoxicants**

Inflammation of the brain, or neuroinflammation, triggered by environmental toxicants, is recognized as a major contributor of PD progression during neurodegeneration. Although divergent molecular events characterize the progression of Parkinson’s disease (PD), Alzheimer’s disease, and amyotrophic lateral sclerosis, neuroinflammation emerges as one common theme among the cellular events underlying these neurodegenerative diseases (Block & Hong, 2005; Heneka et al., 2015; J. K. Lee, Tran, & Tansey, 2009; McGeer & McGeer,
2004; Mrak & Griffin, 2005). Recent work from animal studies and epidemiological studies have provided evidence that neuroinflammation contributes to progressive cell death events (Y. S. Kim & Joh, 2006; McGeer & McGeer, 2008).

Neuroinflammation is mediated primarily by two types of glial cells: microglia and astrocytes. Normally quiescent in a healthy brain, glia are immune effector cells that provide critical support for the neurons which they surround. Resting microglia secrete low amounts of pro-inflammatory cytokines and exhibit a ramified morphology, a characteristic of “surveilling” microglia. Similarly, astrocytes normally participate in the glutamine-glutamate cycle, maintain glial-neuron contact, and secrete neurotrophic factors. However, upon toxicant insult, both can be activated. Microglia undergo morphological changes into an amoeba-like morphology, and dramatically increase the release of pro-inflammatory cytokines. Astrocytes upregulate surface expression of GFAP, and secrete pro-inflammatory cytokines. Activation of glia is one of the earliest response to injury that is intended to be a compensatory, neuroprotective response to neurodegeneration as evident by glial release of neurotrophic factors during early phase of injury, and is intended to facilitate phagocytic removal of dead cells or debris or preserve neuronal survival.

However, chronic injury, as can be caused by repeated exposure to environmental toxicants, trigger glial activation that could establish a self-sustaining cycle of neuroinflammation (Orr, Rowe, & Halliday, 2002), which manifests itself as proliferation of activated microglia and astrocytes, activation and nuclear translocation of NF-κB and elevation of cytotoxic cytokines, including tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS), nitric oxide (NO), interleukin (IL)-1β, IL-6, cyclooxygenase-2(COX-2), and prostaglandins E2 (PGE2).
Potent inducers of neuroinflammation, such as MPTP, could elevate brain inflammatory factors years after a single administration in nonhuman primates, long after initial injury and cell death (Orr et al., 2002; Tansey, McCoy, & Frank-Cannon, 2007), suggesting that a cascade of events involving neuronal cell death could aggravate glia activation and produce a vicious cycle of self-propelling and self-sustaining neurotoxicity. In the case of PD, sustained neuroinflammation in the substantia nigra and elsewhere contribute to disease progression, as it is detrimental to dopaminergic neuron survival. Postmortem brains of AD, PD, and HD patients have revealed extensive neuroinflammation with distinct elevation of pro-inflammatory cytokines TNF-α, IL-12, IL-6, and IL-1β. While each of the chemicals described below (manganese, MPTP, rotenone, and dieldrin) may impact cellular processes through unique mechanisms, a common pathway of toxic neuroinflammation emerges.

**Mn**

Manganese (Mn) is an essential trace element in all known living organisms for such fundamental cellular processes as fat and carbohydrate metabolism, regulation of blood sugar, and calcium absorption (Erikson et al., 2005). Mn has a wide range of industrial uses, and is present in welding fumes, mining dust, and fungicides. Risks of its occupational overexposure are therefore increased for welders (Ngwa, Kanthasamy, Jin, Anantharam, & Kanthasamy, 2014), miners (Gendelman et al., 2015), or famers who are exposed to Mn-based pesticides Maneb (Mn ethylene-bis-dithiocarbamate) and Mancozeb. Mn is transported into neuronal cells by divalent metal transporter 1 (DMT1), and chronic overexposure to Mn could directly cause neuronal cell death (Roth, Horbinski, Higgins, Lein, & Garrick, 2002). Mn³⁺ participate
in the oxidation of dopamine to aminochrome (dopaminochrome), the precursor of neuromelanin. However, aminochrome accumulation in cell culture medium induces acute cell death (Paris & Segura-Aguilar, 2011). In neuronal cell cultures, Mn treatment induced cytochrome C release, caspase-3 activation, and DNA fragmentation. More recently, mechanistic studies had shown that caspase-3 activation following Mn treatment could proteolytically activate protein kinase Cdelta (PKCδ), a noncanonical member of protein kinase C family of kinases(Masashi Kitazawa et al., 2005). Cultured neurons expressing a dominant negative form of PKCδ protein were found to be resistant to Mn-induced apoptosis. Small interfering RNA suppression of PKCδ expression or cotreatment with the PKCδ inhibitor rottlerin significantly blocked Mn-induced DNA fragmentation, suggesting that activation of PKCδ signaling represents a major mechanism in Mn-induced apoptotic cell death (Latchoumycandane et al., 2005). Mn could directly cause neuronal cell death, while at the same time also induce neuroinflammation (Sarkar et al., 2018). In astrocyte cultures, Mn exposure increased expression of GFAP, a marker for activated astrocytes, and stimulated the release of proinflammatory cytokines. Mn also disrupts astrocytic regulation of glutamate by increasing the promoter activity of Ying Yang 1, (YY1), a negative regulator of glutamate transporter (GLT-1) (Karki et al., 2014). Microglial activation in response to Mn is even more pronounced. In primary and immortalized microglia cultures, manganese could induce much higher excessive expression of TNF-α, IL-12, IL-6, IL-10, and IL-1β and stimulate iNOS protein levels through the activation of the NF-κB and p50/p65 nuclear translocation to induce expression of inflammatory genes (Filipov, Seegal, & Lawrence, 2005; Verina, Kiihl, Schneider, & Guilarte, 2011). These toxic inflammatory factors are directly cytotoxic to neurons (Filipov et al., 2005)
**MPTP/MPP(+)**

MPTP is a contaminant during the synthesis of meperidine (pethidine), an opioid drug used illicitly. Users who accidently injected MPTP developed irreversible PD-like symptoms of rigidity and bradykinesia (J. Langston, Ballard, Tetrud, & Irwin, 1983). Postmortem analysis of these patients showed selective dopaminergic cell death and marked increase in glial activation in the substantia nigra (J. W. Langston et al., 1999). As a lipophilic pro-toxicant, MPTP readily crosses the blood brain barrier, where it is metabolized by monoamine oxidase B (MAO-B) in astrocytes into active toxicant MPP(+) (Heikkila, Manzino, Cabbat, & Duvoisin, 1984, 1985). The exact mechanism of MPP(+) release from astrocytes is not clear, but nonetheless, it can be taken up by dopaminergic neurons via the dopamine transporter (DAT) (Gainetdinov, Fumagalli, Jones, & Caron, 2002). MPP(+) accumulates in the mitochondria to inhibit complex I of the electron transport chain, thereby causing a dissipation of mitochondrial potential, a collapse in ATP production, and neuronal cell death. Surface expression of signals by dying neurons to stimulate phagocytosis by microglia leads to greater infiltration of the region by activated microglia to remove dead neurons (Tansey et al., 2007).

In microglia, MPP(+) can be taken up via organic cation transporter 3 (OCT3) and potentiates LPS-induced TNF-α expression (Qian He, Wang, Yuan, & Wang, 2017). MPP(+) downregulates miR-7116-5p in microglia, which normally suppresses overproduction of TNF-α, suggesting that MPP(+) -induced dysregulation of this suppression sends TNF-α production in microglia into overdrive (Qian He et al., 2017).

MPP(+) in astrocytes activates inflammasomes, further aggravates neuroinflammation (Qiao et al., 2016). Mechanistically, MPP(+) downregulates ATP13A2, thereby increasing
lysosomal membrane permeabilization and cathepsin B release. This induces activation of nod-like receptor protein 3 (NLRP3) inflammasome, to produce excess IL-1β from astrocytes (Qiao et al., 2016). Furthermore, MPTP treatment reduced IGF-1 levels in the substantia nigra of aged rats (Labandeira-Garcia, Costa-Besada, Labandeira, Villar-Cheda, & Rodríguez-Perez, 2017), which reduced the stimulation AKT phosphorylation and activation (Madathil et al., 2013). When optimally activated, AKT signaling leads to a robust anti-inflammatory response by inducing DJ-1 and HIF1alpha levels (Jha, Jha, Kar, Ambasta, & Kumar, 2015). Disruption of this pro-survival, anti-inflammatory signaling pathway compounds the inflammatory effects produced by glial cells.

**Rotenone**

Rotenone is a broad-spectrum insecticide, piscicide, and pesticide, and exposure to it has been implicated in the development of neuroinflammation and the progression of PD (Betarbet & Greenamyre, 2008; Cannon et al., 2009; Dranka, Zielonka, Kanthasamy, & Kalyanaraman, 2012; Sherer, Betarbet, Kim, & Greenamyre, 2003; Tanner et al., 2011). Rotenone is a known inhibitor of mitochondrial complex I; its exposure in neuronal cells causes the collapse of mitochondrial potential and release of mitochondrial cytochrome C, initiating the intrinsic apoptotic pathway. In microglia, rotenone exposure directly induces the phosphorylation of p38 and activation of p38 MAPK pathway, a stress activated protein kinase pathway that leads to NF-κB activation and p50/p65 transcription factor nuclear translocation for expression of pro-inflammatory genes IL-1beta and TNF-α (Bachstetter et al., 2011). Although the role of NF-κB remains controversial, activation of NF-κB in microglia and astrocytes generally results in production of proinflammatory cytokines TNF-α and IL-6,
which are produced in particularly high amounts by glial cells (Mattson & Camandola, 2001). In addition, transcription factor p65 can bind to the NF-κB consensus sequence on the COX-2 promoter, leading to expression of COX-2, a major pro-inflammatory mediator (Minghetti, 2004).

Rotenone exposure also induces the activity of GSK3β (Han, Casson, Chidlow, & Wood, 2014), a crucial regulator of the inflammatory response. GSK3β has an inhibitory effect on CREB nuclear translocation, thereby allowing for transcription of pro-inflammatory genes such as interleukin-1-Beta (IL-1β) and TNF-α(Maixner & Weng, 2013). Inversely, inhibition of GSK3β increases CREB DNA binding activity and increases transcription of anti-inflammatory IL-10 (Maixner & Weng, 2013). Rotenone-induced cytotoxicity in cultured dopaminergic neuronal cells could be attenuated by the GSK3β inhibitor SB216763 (Hongo et al., 2012), suggesting that GSK3β signaling could mediate rotenone-induced neuronal toxicity. In astrocytes, GSK3β induction generally increases iNOS, nitric oxide (NO), cyclooxygenase-2 (COX-2), prostaglandins E2 (PGE2), and TNF-α expression (H.-M. Wang et al., 2013).

In microglia, GSK3β mediates the increased release of pro-inflammatory cytokines (Q. Cao, Karthikeyan, Dheen, Kaur, & Ling, 2017). A general mechanism could be mediated through activation of mixed lineage kinases (MLK). Both rotenone and MPP(+) can activate GSK3β, and decreasing GSK3β activity blocked MLK3 signaling cascades through disruption of MLK3 dimerization-induced autophosphorylation, inhibiting the downstream stress activated JNK pathway, ultimately leading to a decrease in TNF-α secretion(L. H. Wang, Besirli, & Johnson, 2004; M.-J. Wang, Huang, Chen, Chang, & Kuo, 2010). Furthermore, the pan-MLKs inhibitor, CEP-1347, was shown to prevent dopaminergic neuronal loss in pre-clinical animal models of PD (A. Kanthasamy et al., 2012).
**Dieltrob**

Dieltrob is an organochlorine pesticide that was used in the US in the late 1980s for control of insects. Despite its discontinuation of use in early 1990s, it persists heavily in the environment. Dieltrob could stimulate pro-inflammatory IL-8 and TNF-α responses in Jurkat cells (Saliha, Eric, Frederique, Etinne, & Christian, 2018), and can activate peripheral neutrophils to promote the production of IL-8 (Pelletier et al., 2001). The highly lipophilic dieltrob readily crosses the blood brain barrier (Moretto & Colosio, 2011) and its exposure has been implicated in PD disease progression (Cowie et al., 2017; A. G. Kanhasamy et al., 2008). Presence of dieltrob could be seen in postmortem analysis of brains in some PD patients but not in control brains (Corrigan, Murray, Wyatt, & Shore, 1998; Fleming, Mann, Bean, Briggle, & Sanche-Ramos, 1994; A. G. Kanhasamy, Kitazawa, Kanthasamy, & Anantharam, 2005), together with high levels of glial activation, thus correlating dieltrob exposure with neuroinflammation. While less is known about dieltrob’s role in induction of neuroinflammation, evidence from in vitro studies so far has shown that dieltrob could activate microglia via NADPH oxidase 2 (NOX2) activation (Taetzsch & Block, 2013) to increase ROS, thereby causing a pro-inflammatory state in microglia. Additionally, dieltrob could activate the non-receptor tyrosine kinase, Fyn kinase (Saminathan, Asaithambi, Anantharam, Kanthasamy, & Kanthasamy, 2011), which, could phosphorylate PKCδ at the Y311 site, resulting in increased PKCδ kinase activity (Panicker et al., 2015). Similar to Mn, dieltrob could also induce neuronal cell death by activating caspase-3, then caspase-3-dependent proteolytic activation of PKCδ (M. Kitazawa, Anantharam, & Kanthasamy, 2003). Furthermore, dieltrob exposure has been found to induce aberrant acetylation of core histone H3 and H4 within minutes of exposure in dopaminergic neuronal cultures (C. Song,
Kanthasamy, Anantharam, Sun, & Kanthasamy, 2010). Alternatively, dieldrin treatment could also induce caspase-dependent proteolytic cleavage and inactivation of poly(ADP-ribose) polymerase (PARP), a cellular pathway for DNA damage repair that can be inactivated by extensive DNA damage and activated by pro-survival BCL-2 overexpression (Masashi Kitazawa, Anantharam, Kanthasamy, & Kanthasamy, 2004). Interestingly, dieldrin exposure-induced epigenetic changes in Jurkat T cells could cause increased transcription of human endogenous retroviruses, vestiges of ancient retroviral infections of the germline normally kept in check by heterochromatin (Saliha et al., 2018). Expression of these degenerated copies of viral genes nonetheless could induce inflammation and is implicated in multiple sclerosis.

**Synergistic Effects**

The cause of neuroinflammation and the resulting neuronal cell death is generally multifactorial, involving exposure to chemicals that could initiate such toxic conditions, or several such initiators that could potentiate neuroinflammatory effects, as well as cellular responses to disturbances of signaling pathways. Bacterial inflammogen LPS has been shown to potentiate neurotoxic effects induced by environmental toxicant rotenone (Gao, Hong, Zhang, & Liu, 2002). Similarly, Mn-containing pesticide Maneb could act synergistically with paraquat, another pesticide with structural similarity with MPTP, to produce compounded toxicity on the dopaminergic system (Thiruchelvam, Brockel, Richfield, Baggs, & Cory-Slechta, 2000). Such multifactorial mode of exposure more realistically reflects the condition experienced in patients during neuroinflammation induced by neurodegeneration.

As mechanisms of action for environmental toxicants to cause neuroinflammation are elucidated, therapeutic strategies are devised against these target pathways. For example, the
Fyn inhibitor saracatinib is used to inhibit Fyn-PKCδ signaling in status epilepticus-induced neuroinflammation (Sharma et al., 2018), and rotenone-induced cytotoxicity in cultured dopaminergic neuronal cells could be attenuated by the GSK3β inhibitor SB216763 (Hongo et al., 2012). Epidemiology studies and animal studies had suggested that non-steroidal anti-inflammatory drugs (NSAIDs) could lower risk of PD (McGeer & McGeer, 2004). Lastly, neuroinflammation could also aggravate tissue damage by inducing oxidative stress in glia, through production of high levels of ROS, and through arachidonic acid signaling by activating cyclooxygenase (COX) and lipoxygenase (LOX) pathways.

**Hypotheses Regarding PD Pathogenesis**

Several hypotheses have attempted to correlate environmental exposures with the development of PD motor and non-motor pathologies.

**Braak staging**

The Braak staging hypothesis attempts to explain PD pathology in terms of PD temporal and spatial progression; it correlates the exposure of environmental agents (chemicals, viruses, bacteria) in the peripheries (nose, gut) with initial appearance of Lewy bodies in the olfactory system and enteric nervous system (Braak et al., 2003; Jellinger, 2009), resulting in gut constipation and lessened sense of smell. Interestingly, a 1918 pandemic flu that became strongly associated with post-encephalitic parkinsonism also had lend strength to this hypothesis (Billingsley et al., 2018). During stage II-III of the Braak staging, Lewy body pathology subsequently enters the central nervous system, and by spreading in a caudal-to-rostral direction, affects the brain stem, followed by substantia nigra, (Dickson, Uchikado, Fujishiro, & Tsuboi, 2010), damaging particularly the non-myelinated dopaminergic neurons.
In later stages of PD, Lewy pathology have been found in the cortex, corresponding to the later Braak stages (Del Tredici & Braak, 2016).

**Oxidative stress and mitochondrial dysfunction**

SNpc dopaminergic neurons are uniquely vulnerable to damage. These non-myelinated neurons, number approximately 300,000-600,000 in humans (Chinta & Andersen, 2005; Schultz, 2007), have extensive innervations extending into the striatum (each neuron has an upwards of 150,000 presynaptic terminals in the striatum (Roberts, Force, & Kung, 2002)), resulting in neuronal soma accounting for only 1% of cell volume (Sulzer, 2007). The extensive networks of innervations require relatively higher energy demands and are thus more susceptible to defects in organelle trafficking. Further, since neuromelanin is a major iron storage in the brain, the highest concentrations of iron are found in the SN and the striatum (Fernandez, Ferrer, Gil, & Hilfiker, 2017). The accumulation of excess iron is further substantially increased in brains of PD patients (Jiang, Luan, Wang, & Xie, 2006). Excessive iron can cause hydroxyl radical production via the Fenton reaction, leading to oxidative stress accompanied by the oxidation and modification of proteins, lipids, carbohydrates and DNA. The oxidative stress is compounded by the spontaneous auto-oxidation of dopamine to produce O2-, which is converted to H₂O₂ (Miyazaki & Asanuma, 2008) in mitochondria of dopaminergic neurons, inducing mitochondrial damage (Surmeier & Schumacker, 2013). The convergence of oxidative stress and mitochondrial dysfunction make SNpc dopaminergic neurons particularly vulnerable. Borrowing a concept from cancer biology where multiple hits by initiator carcinogens, promoter carcinogens, and loss of apoptotic signal balances are required for cancer progression and metastasis, the multiple hit hypothesis for PD etiology
focuses on environmental exposure as “primary hits” that initiate neuronal stress in combination with loss of protective pathways in neurons as “secondary hits”, resulting in dopaminergic neurodegeneration (Sulzer, 2007).

**Olfactory vector hypothesis**

Another hypothesis, the olfactory vector hypothesis for PD, attempts to correlate olfactory deficits, a symptom manifested in almost 90% of both Alzheimer’s disease (AD) and PD during early stages of these diseases (Richard L. Doty, 2008), with the initiation of AD and PD pathologies (R L Doty, Reyes, & Gregor, 1987; Ward, 1986; Wattendorf et al., 2009), with some proponents even suggesting that PD is primarily an olfactory disorder with accompanying motor dysfunctions during later stages (Hawkes, 1999). This hypothesis for PD pathogenesis takes into consideration that 1) clinical observation of PD found olfactory deficits “rivals or exceeds the prevalence rate of the defining motor signs of the disorder”; 2) findings that suggest pesticide exposure is a top risk factor for PD; 3) a number of viruses, aerosolized metals, chemicals, could be taken up by olfactory sensory neurons and enter the brain through the olfactory bulb, a circumventricular organ that allow toxicants to bypass much of the blood brain barrier. A number of studies indicating that MPTP treatment or LPS treatment administered through the nasal cavity better recapitulate motor and non-motor symptoms compared to intraperitoneal administration seem to support this hypothesis (D. S. Prediger et al., 2011; Qing He et al., 2013).
Animal Models of PD for Studying Gene-Environment Interactions

Several animal models of PD have been developed in order to test hypotheses regarding PD pathogenesis and to evaluate therapeutic strategies devised against PD progression. MPTP is the most frequently used Parkinsonian toxicant applied in the generation of animal models of PD (Beal, 2001; Przedborski et al., 2001), with the obvious advantage that of MPTP was clinically observed in 1979 to produce a human model of the disease upon accidental injection (Davis et al., 1979; J. Langston et al., 1983).

More recently, a genetic model of PD called MitoPark mice has been developed which recapitulate most of the characteristic behavioral symptoms of PD, including the slow progressive dopaminergic degeneration that takes place over a time course of months. This causes a gradual onset of motor function impairment seen in PD patients (Ekstrand et al., 2007). The animal model is constructed by conditionally knockout of mitochondrial transcription factor A (TFAM) in dopaminergic neurons using the Cre-loxP system, leading to reduced mitochondrial DNA expression, and respiratory chain deficiency (Ekstrand et al., 2007). Interestingly, using MitoPark mice, it was found that Mn exposure worsened depletion of striatal dopamine and accelerated the progressive nature of motor deficits already taking place. Importantly, using the MitoPark mice, Mn was found to aggravate the neuroinflammatory processes as indicated by increased IBA-1-immunoreactive microglia cells in the SN, strengthening the experimental evidence the interaction between PD susceptibility genes and environmental exposures. The MitoPark mouse genetic model of PD therefore represent a unique model for studying the interaction of genetic and environmental factors, and their contributions to the neurodegenerative process, during disease progression (Langley et al., 2018).
Background and Literature Review II

Current PD Experimental Therapy Landscape and the Potential of GDNF

PD remain an incurable disease condition and the state-of-the-art PD therapy remain focused on disease symptomatic management (Pires et al., 2017). L-DOPA, the precursor for dopamine, was discovered by Swedish scientist Arvid Carlsson in the 1950’s to mitigate motor symptoms in Parkinsonian mice, seminal findings to which the Nobel Prize in physiology or medicine was awarded in 2000. Decades since discovery of L-DOPA’s effects, it remains the strongest arsenal against PD disease symptoms (Mercuri & Bernardi, 2005). Currently, pharmacological remediation for countering the decrease in dopamine concentrations or receptor activity in the caudate putamen include dopamine agonists and monoamine oxidase B (MAO-B) inhibitors and Catechol-O-methyl transferase (COMT) inhibitors, both which inhibit degradation of dopamine, and are used many times in combination with L-DOPA administration.

Although more choices in medications and combinations of medications have been recently made available, the fundamental treatment strategy of symptomatic management has remained the same. Despite an increasingly complex combination of therapies, once the “honeymoon” of L-DOPA period has waned, PD patients derive increasingly less efficacy (Rascol et al., 2003) and increasingly more side-effects (Smith, Wichmann, Factor, & Delong, 2012) from the available treatments. Chronic use of L-DOPA leads to dyskinesia induced by stimulation of dopamine receptor 1 expressed by medium spiny neurons in the striatum. To mitigate dyskinesia induced by L-DOPA treatment, dopamine receptor agonists apomorphine, pramipexole and ropinirole are prescribed, either in place of L-DOPA, or more likely during later stages of PD, in combination with L-DOPA (Holloway et al., 2004). However, although
they reduce dyskinesia, they also increase orthostatic hypotension and psychiatric symptoms such as psychosis, depression. In the case of orthostatic hypotension, it affects 30% of the PD patients and is treated with L-Threo-dihydroxyphenylserine (L-DOPS) (S., M.V., A., & O., 2013). In the case of PD-associated psychosis, it affects around half of advanced PD patients, and limited options include pimavanserin (Holloway et al., 2004). Surgical interventions for PD symptomatic control involves the implantation of deep brain stimulation (DBS) device into the brain of PD patients, commonly the internal pallidum or the subthalamic nucleus (Fang & Tolleson, 2017). An electrical wire is planted inside the target area, which applies intermittent direct electrical current, with frequencies above the native firing rates, thus generating an inhibitory effect on the target (Benazzouz & Hallett, 2000). However, major drawbacks, aside from being only available to selective patients, include surgical complications, occasional hardware failure, and worsening of neuropsychiatric side effects (Ughratdar, Samuel, & Ashkan, 2015). In late stage PD, treatment-resistant motor symptoms contribute to the burden of complications facing PD patients, for whom falls or accidental choking could be common (Hely, Morris, Reid, & Trafficante, 2005). Clearly, the current challenge is a fundamental treatment therapy that could slow or stop the progressive nature of the disease and restore dopaminergic innervation and function.

**Current Clinical Trials for Experimental Therapies**

Current disease-modifying clinical trials focus on three primary goals, which are (1) to compensate for dopamine deficiency or other neurodegenerative changes; (2) to provide trophic support to neurons and compensate for metabolic abnormalities; and (3) to replace lost dopaminergic neurons using cell-based therapies (Lang & Espay, 2018). A summary of clinical trials currently underway is listed in Table 2 (Athauda & Foltynie, 2016; Bergstrom, Kallunki,
As a hallmark during PD neurodegenerative process, alpha-synuclein aggregation has been the target of a number of clinical trials, with goals for its degradation or reduction of its protein synthesis, misfolding, and cell-to-cell transmission (Kalia, Kalia, & Lang, 2015). However, instead of focusing on early events, a critical downside to these clinical trials lie in that they focus on the formation of alpha-synuclein aggregation and Lewy body, a late-stage
event in the pathophysiology of PD. Moreover, since alpha-synuclein has been found to protective during early stage of disease, more concrete understanding of the role of alpha-synuclein is needed to ensure successes of therapies targeting alpha-synuclein. Some studies focus on specific aspects of the neurodegenerative process as clinical endpoints, rather than improvements in patient UPDRS, for demonstration of efficacy. For example, AZD3241 is being evaluated in a phase IIa randomized placebo controlled multicenter positron emission tomography (PET) study for its effects on activated microglia in PD patients (Jucaite et al., 2015). EPI-589 (R-troloxamide quinone) is being evaluated in phase II trial using the increases in cellular levels of antioxidant GSH as a biomarker and endpoint (clinical trial NCT02462603). Aside from ones listed in the table, Epigallocatechin gallate (EGCG) is also nearing clinical trial stage (Perni et al., 2017; Yan Xu et al., 2016). Lastly, glial cell-derived neurotrophic factor (GDNF) which has been evaluated in a number of high profile clinical trials but did not realize hoped-for results. It is currently under a phase II trial in association with the Michael J. Fox Foundation using an improved delivery method. The potential of GDNF as a therapeutic candidate is a topic which will receive special attention in the following sections.

**Glial-cell line Derived Neurotrophic Factor**

GDNF is the founding member of GDNF family of neurotrophic factors artemin, persephin and neurturin, which are members of the transforming growth factor-β superfamily (Airaksinen & Saarma, 2002). GDNF is a glycosylated homodimer, with a molecular weight of 33-45 kDa as a dimer and 16 kDa after deglycosylation (L. F. Lin et al., 1993). Synthesized as a 211-amino acid pre-proGDNF, it is cleaved by furin proteases prior to secretion as a 134-amino acid mature protein.
Each of the four members of the GDNF family signal through multicomponent receptor complexes, by preferential binding of GDNF, neurturin, artemin, or persephin to glycosylphosphatidylinositol-anchored cell surface protein GFRα1, GFRα2, GFRα3, GFRα4, respectively. The GDNF-GFRα1 complex binds to the Ret tyrosine kinase, the GDNF co-receptor, for transduction of intracellular signaling (Figure 1).

Figure 1. Signaling mechanisms of GDNF family neurotrophic factors. GFRα receptors are preferentially located on lipid rafts, and the pairs GDNF-GFRα1, neurturin-GFRα2, artemin-GFRα3, persephin-GFRα4 activate co-receptor Ret tyrosine kinase for signal transduction. Adapted from (Airaksinen & Saarma, 2002)

**Regulation of GDNF expression**

A number of factors could induce expression of GDNF, such as docosahexaenoic acid, imipramine, adenosine, apomorphine, dopamine, and riluzole (Y. Kim et al., 2011; L. Zhang et al., 2017). During development, GDNF support the growth of midbrain dopamine neurons and motoneurons, as well as peripheral neurons, including sympathetic, parasympathetic,
sensory and enteric neurons (Sariola, 2003). A number of transcription factors, Pax2, Eya1, Six1, Six2, Sall1, Foxc1, Wt1, and Hox11 could induce GDNF expression during kidney development. (Saavedra, Baltazar, & Duarte, 2008).

During neuronal injury, the predominant amount of GDNF is secreted by glial cells for neuronal maintenance and survival (Saavedra et al., 2008). Compensatory induction of GDNF is observed in response to lipopolysaccharide (LPS) treatment in astrogliaoma cells (Appel, Kolman, Kazimirsky, Blumberg, & Brodie, 1997). In the dopaminergic system, Ret expression is required for modulation of protective effects, whereas the absence of Ret abolishes GDNF's neuroprotective and regenerative effect (Drinkut et al., 2016).

In hippocampal and cortical neurons lacking Ret tyrosine kinase, the neuronal cell adhesion molecule (NCAM) is an alternative signaling receptor for GDNF family neurotrophic factors. GDNF-GFRα1 association with NCAM, instead of Ret, induces axonal growth in hippocampal and cortical neurons via binding to NCAM, leading to its interaction with focal adhesion kinase Fak (Paratcha, Ledda, & Ibáñez, 2003). Transcriptionally, GDNF can be upregulated by Nurr1, Pitx3 in cultured dopaminergic neurons. Activation of p44/42 ERK and PI3-K/AKT pathways have also been show to induce GDNF expression (Tanabe, Matsushima-Nishiwaki, Iida, Kozawa, & Iida, 2012). GDNF can also be regulated by FGF2, via induction of EGR1 (Shin et al., 2009). Epigenetically, chronic stress could affect histone modifications and DNA methylation of the Gdnf promoter, leading to changes in GDNF expression (Uchida et al., 2011).

GDNF is currently being assessed for its therapeutic potential in clinical trials, as detailed in the next sections. As one of the most studied neurotrophic factors in the brain, much knowledge has accumulated about its biology and its mechanisms of action. However,
transcription factors that drives its expression are not completely known, and even less is known about pharmacological agents that could modulate its expression. Yet, such agents could prove immensely helpful in activating GDNF or other neurotrophic factors in brains of PD patients.

**GDNF: a promising candidate for disease modifying therapy**

*Discovery and early studies*

With early studies in 1993 that discovered GDNF in glioma cell lines, researchers quickly realized its potential as a potent and selective growth factor for mesencephalic dopaminergic neurons and a stimulator of neurite growth (L. F. Lin et al., 1993). By 1994, direct injection of GDNF had been used in unilateral 6-OHDA rat model of PD for its effects on amphetamine-induced contralateral rotational behavior, and showed a 4-fold decrease in contralateral rotational behavior in rats that received ipsilateral dopaminergic neuron degeneration (Hoffer et al., 1994). The next year, in 1995, studies injecting GDNF in the SN or in striatum of mice before MPTP injection found potent protection against MPTP-induced nigrostriatal lesion, and observed higher dopamine nerve terminal densities and dopamine levels (Tomac et al., 1995). By 1996, monkeys treated with MPTP were used to successfully show the potential of intraputamental injection of GDNF in both rescuing motor deficits and restoring striatal dopamine levels (Gash et al., 1996).

*First-in-human safety and tolerability studies (Rush Medical Center Chicago)*

Perhaps emboldened by the non-human primate study, the first-in-human study was done at Rush Medical Center in Chicago in 1999, led by Jeffrey Kordower, in a 65-year-old
PD patient, who received monthly intracerebroventricular injections of GDNF (J H Kordower et al., 1999). However, no improvements on motor symptoms were seen, and side effects included nausea, loss of appetite, tingling, intermittent hallucinations, and depression. Importantly, GDNF was thought to not have diffused into the deep layers of the brain (intraparenchymal diffusion). The results suggested that an alternative GDNF delivery system is needed. A second study in monkeys was reported in 2000, where lentivirus coding for GDNF was injected into the striatum and SN of young adult rhesus monkeys treated prior with MPTP. The lenti-GDNF was able to reverse functional deficits and completely prevented nigrostriatal degeneration (J. H. Kordower et al., 2000). The study had also found extensive anterograde and retrograde transport of GDNF, and it was realized later that GDNF retrograde transport to the SN was required for its protective effects (Tenenbaum & Humbert-Claude, 2017). A third study in 2002 subjecting MPTP-treated rhesus monkeys with chronic infusion of 5 or 15 µg/day of GDNF using programmable pumps into the lateral ventricle or the striatum, had found restoration of the nigrostriatal dopaminergic system and significantly improved motor functions (Grondin et al., 2002).

*Intracerebroventricular GDNF injection (Phase I/II, open-label, led by Gary Nutt)*

Prompted by new efficacy data in primates, in the same year in 2002, a prominent Swedish neuroscientist, Patrick Brundin, called for a clinical trial evaluating GDNF as a PD therapy (Brundin, 2002). In a perhaps still premature attempt, the first controlled trial was conducted in 2003, led by Gary Nutt, to test the safety, tolerability, and biological activity of GDNF administered by an implanted intracerebroventricular (ICV) catheter in a dose-escalation study in 50 PD patients for 8 months (Nutt et al., 2003). After the 8 months, some
patients became part of the open-label study extending exposure up to an additional 20 months, and some patients were in groups that received the maximum of 4,000µg GDNF. Unfortunately, the study did not achieve the efficacy that was hoped: motor UPDRS scores were not improved, and moreover, significant adverse effects, such as nausea and vomiting were common hours to several days after treatment with GDNF, and one person died after three weeks, from events unrelated to GDNF treatment.

*Direct putamenal GDNF injection (Phase I, open-label, Frenchay Hospital)*

Since the first-in-human study reported in 1999 had speculated that ICV-administered GDNF did not penetrate deep into the layers of brain into the SN and striatum, another study, led by Steve Gill, had reported in 2003 which tested safety and feasibility of direct putamenal injection of GDNF protein using catheters in 5 PD patients (Gill et al., 2003). The recombinant methionine human GDNF was produced by Amgen as liatermin or r-metHuGDNF, and had the sequence of mature GDNF with an N-terminal-tagged methionine. The results from this open label, phase I safety study were positive, with no serious clinical side effects and drastic improvements in motor symptoms. Positron emission tomography (PET) scans of [(18)F]dopamine uptake also showed improvements in putamen dopamine storage after 18 months. However, since this study was an open label study, a randomized, controlled study is needed.

*Intraputamenal GDNF infusion (Phase II, randomized controlled trial, Amgen)*

Encouraged by results from previous open-label study, a phase II trial started in 2003 and reported in 2006, led by Anthony E Lang at the Toronto Western Hospital, in association
with Amgen. A randomized controlled trial of 34 PD patients receiving continuous bilateral intraputamenal infusion of liatermin 15 at µ/day, or placebo, in each putamen, was conducted (Lang et al., 2006). Although patients did not develop severe side effects, no change in primary endpoint was seen and no change in UPDRS ratings were found. Additionally, some patients developed antibodies against GDNF. Although are no direct adverse effect from production of antibodies against GDNF during the treatment course, the neutralizing antibodies could render the treatment less effective, and depending on the epitopes that the antibodies targeted, could further decrease endogenous GDNF after treatment cessation.

Unilateral intraputaminal GDNF infusion (Phase I, open-label, led by John Slevin)

Another study was done in parallel for testing the safety and tolerability of a dose-escalation regimen of administration of Amgen’s GDNF protein, increasing from 3 µg/day, to 30µg/day, over a 6-month period. The delivery method used was unilateral intraputaminal GDNF infusion using a multiport catheter that delivered continuous infusion at 2µl/h, into the most affected putamen in the 10 patients with advanced PD (Slevin et al., 2005). At 24 weeks of treatment, UPDRS scores significantly improved, as did balance and gait. The only observed side effects were transient Lhermitte's sign, a tingling electric shock sensation produced by flexing the neck. The open-label study demonstrated the safety and potential efficacy of unilateral intraputaminal GDNF infusion and showed sustained effects after the withdrawal of treatment. But before a phase II trial could be conducted, Amgen halted all trials using GDNF, since the earlier phase II trial led by Anthony Lang did not meet primary endpoint of improvement in UPDRS, and toxicity in higher doses of GDNF was seen in non-human primates. A follow-up study of patients in the phase I open-label trial confirmed the previous
phase II trial; all benefits seen during treatment were lost after 1 year of GDNF infusions, UPDRS returned to patient baseline, and motor symptoms required higher levels of conventional antiparkinsonian drugs (Slevin et al., 2007). Further, GDNF neutralizing antibodies were also detected.

**GDNF family - Neurturin phase I/II trials**

Another GDNF family member, neurturin, was undergoing a number of clinical trials in parallel with GDNF. In 2008, the first safety and tolerability of intraputaminal delivery of adeno-associated virus serotype 2-neurturin (AAV2-neurturin/CERE-120) was conducted in PD patients by the pharmaceutical company Ceregene (Marks et al., 2008). In this open-label trial, the delivery method was found safe and feasible. But two years later, in 2010, Ceregene reported that the a double-blind, randomized, controlled trial of CERE-120 largely did not meet its primary endpoint (Marks et al., 2008), and importantly, brain tumors were found in both the AAV-neurturin treatment group and the AAV vector group, suggesting that the vector could be associated with tumorigenesis.

Since the report also suggested that impaired axonal transport of neurturin from putamen to SN reduced the effectiveness of neurturin injection in striatum, SN-injection of AAV-neurturin was therefore assessed for functional efficacy in rats (Herzog et al., 2013), then in human (Bartus et al., 2013) for safety and feasibility of direct delivery into the SN, which reported in 2013. For a second double-blind, randomized, controlled trial reported in 2015 (Warren Olanow et al., 2015), AAV-neurturin or placebo were delivered into both putamen and SN in 51 PD patients. Although there were no clinically meaningful adverse events, no significant differences were found between the groups in the evaluation of primary endpoint.
Despite the promising potential of GDNF and GDNF family member neurturin, side effects and efficiency in delivery remain big hurdles. To date, all attempts to develop GDNF or any other therapy as a cure for PD have failed. Candidate therapies shown to work in current animal models of PD had failed to modify the progression of disease and show improvements in human clinical endpoints. Two major aspects that undermined the efficacy of GDNF in humans have been attributed to difficulty in candidate drug evaluation in animal models and delivery of the drug into patients.

*Reasons for trial failures: animal model selection*

Currently, no single animal model of PD exists that is ideal for all types of studies. Administration of Parkinsonian toxicants such as 6-hydroxydopamine (6-OHDA) and MPTP in rodents and primates have been widely-used to elicit dopaminergic neuronal degeneration and motor symptoms, and have been tremendously useful in evaluating dopamine-based therapies that could potentially provide symptomatic management (Bezard, Yue, Kirik, & Spillantini, 2013; C. Warren Olanow & Kordower, 2009). However, dopamine-based therapies are themselves stopgap solution in finding a disease-modifying therapy.

The MPTP model is the most commonly used model, and the most commonly used method employs acute administration of MPTP into animals to selectively kill dopaminergic neurons. However, in PD patients, dopaminergic neuronal death occurs in a backdrop of a slowly evolving neurodegenerative processes, with competing compensatory responses, and the acute administration of MPTP may not properly reflect the time-dependent, complex cascade of events relevance to what occurs in the brain of a PD patients. Instead, MPTP treatment in mice could have its own compensatory response different from that of PD patients.
For example, acute MPTP treatment could induce TH+ neurons to increase their branching as a compensatory response (D. D. Song & Haber, 2000).

To reduce attrition of drug candidates, more accurate animal models would need to be employed to evaluate new neuroprotective therapies. To this end, transgenic models based on genes involved in the pathogenesis of PD found through human genetic studies have increasingly received more attention.

*Reasons for trial failures: delivery methods and side effects*

Purified GDNF protein was directly injected into the putamen of PD patients in the trials conducted by Amgen. In a separate study using the same protocol and procedures, Salvatore et al. had reported that unilaterally infused GDNF in the putamen of adult rhesus monkeys was concentrated around the delivery catheter, with concentration dropping exponentially in the tissues away from the catheter (Salvatore et al., 2006). The volume of distribution of GDNF around the catheter ranged between 87 to 369 mm$^3$, which covers 2%-9% of the total 3-4000 mm$^3$ of the human putamen (Salvatore et al., 2006). Since GDNF protein has a limited diffusion range, an ideal delivery method, aside from pharmacological modulation of endogenous GDNF, would carry GDNF or GDNF-coding cDNA further away from the delivery site, into larger areas of the putamen and SN, for efficient protective effects. Although AAV2-mediated delivery of GDNF cDNA allow GDNF expression in wider areas of the putamen, as seen in the AAV2-neurturin trial, both AAV2-neurturin and vector were associated with tumorigenesis (Marks et al., 2008). Lastly, some patients had developed GDNF-neutralizing antibodies. Little is known about this process, but it is likely that excess GDNF injected into the brain is leaked from the CNS into the peripheries, where the exogenous
GDNF protein encounters immune cells in the peripheries. In summary, for the above reasons, pharmacological modulation of endogenous GDNF levels that could be tightly controlled might resolve issues associated with protein diffusion, leakage, and transgene tumorigenicity.

**Background and Literature Review III**

**Prokineticin-2**

**Structure of Prokineticins**

Prokineticin-1 and prokineticin-2 are a pair of secreted signaling neuropeptides discovered around thirty years ago. From the venom of the black mamba snake, a non-toxic cysteine-rich protein was isolated and named mamba intestinal toxin 1 (MIT-1), for its ability to potently contract guinea pig ileum (Schweitz, Pacaud, Diochot, Moinier, & Lazdunski, 1999). Later it was found to be homologous with another protein isolated from the skin of the frog Bombina variegata named Bv8 (Mollay et al., 1999). Since both could potently induce gut motility, when the human homologs were cloned, the MIT-1 homolog was named Prokineticin-1 (PK1), which maps to chromosome 1p21. The Bv8 homolog was named Prokineticin-2 (PK2), which maps to chromosome 3p21.1, an unstable chromosomal synteny breakpoint region. PK1 (86 amino acids), and PK2 (81 amino acids) share 45% amino acid identity. Contained within the protein sequences are 19 peptides serving as secretion signal in the mature proteins.

Prominent structural features and determinants include 10 crosslinked cysteine residues forming five disulfide bridges, which is important for the structural integrity of mature PK1 and PK2, as highlighted in purple in Figure 2. The mature PK1 and PK2 proteins are predicted to have an approximate ellipsoid shape. Receptor binding are thought to involve the exposed
ends of N-terminus and C-terminus, of which the conserved N-terminus sequence of 6 amino acids AVITGA, and the C-terminal cysteine-rich domain, are indispensable for functional activation of the receptors (Figure 2). Mutations in the AVITGA sequence could abolish its activity, and substitution of alanine for methionine at the N-terminal produce PKR1 and PKR2 antagonists. However, the AVITG peptides alone cannot activate the receptors (Bullock, Li, & Zhou, 2004). Mutations in cysteine residues of the C-terminal domain also result in PK1 and PK2 without biological activity (Bullock et al., 2004).

A PK2 splice variant containing an extra 21 amino acids between exons 2 and 4, named PK2L, could undergo furin proteolytic cleavage of to produce PK2β, which has roughly 10-fold lower potency in binding to PKR1 or PKR2 as compared to PK2, and showed selectivity for PKR1 (J. Chen et al., 2005). The function of the splice variant in PK2 signaling is still unclear. No splice forms of PK1 are currently found. The isoelectric point (pI) of PK2 is a relative basic 8.85, and pI of PK2L is 10.68, due to its extra 21 amino acids that are rich in lysine and arginine.
Figure 2. Multiple sequence alignment of mature protein sequences for human PK1 (red), human PK2 (yellow), frog PK2 homolog Bv8 (green), and snake PK1 (blue) reveals 10 conserved cysteines in PK1 and PK2. PK1 and PK2 have 44% amino acid identity. The N-terminal sequences (yellow highlighted region) before the first cysteine (AVITGA) is conserved among all species.

**PK1 and PK2 Signaling Through PKR1 and PKR2**

PK1 and PK2 bind to two cognate receptors prokineticin receptor 1 (PKR1), and prokineticin receptor 2 (PKR2) with similar affinity (D. C. Lin et al., 2002). The non-mammalian homolog of PK1, MIT-1, is a PKR2-prefering agonist (L. Negri & Ferrara, 2018). In humans, PKR1 and PKR2 are located at chromosome 2p13.3, and 20p13, respectively. The prokineticin receptors are G protein-coupled receptors (GPCR), belonging to the family-A of GPCR, and are related to the neuropeptide Y receptor family, which has been found to stimulate migration of neuroprogenitor cells in the SVZ (Decressac et al., 2009). The sequences of both receptors are highly conserved, with nearly 85% identity, with most of the sequence variation appearing in the extracellular N-terminal region (D. C.-H. Lin et al., 2002). The structure of PKR1/PKR2 is shown in Figure 3. The endogenous peptide ligands PK1 and PK2 make contacts on the extracellular surface of the receptors with the second extracellular
loop, which is stabilized by a disulfide bond formed between two cysteine residues located on the first and second extracellular loops (L. Negri & Ferrara, 2018).

Figure 3. PKR1 and PKR2 have 85% sequence similarity. The predicted structure of PKR1 and PKR2 (differences shaded in black). The extracellular domain (N-terminus) and intracellular (C-terminus) are labelled. Seven transmembrane regions are shaded in grey boxes.

The binding of PK1 or PK2 to either receptors may be coupled to Gq to induce calcium mobilization (Q Y Zhou, 2006), or Gs to induce cAMP accumulation (J. Chen et al., 2005), or Gi to activate p44/42 MAPK signaling (Ngan & Tam, 2008), indicating that multiple pathways are involved in prokineticin signaling depending on cell type. Figure 4 provides an overview of prokineticin signaling in the body primarily outside of CNS.
Figure 4. Summary of Prokineticin signaling in the body. Particularly in the enteric system, Prokineticin signaling could crosstalk with GDNF signaling to induce differentiation, and migration of ENS neurons. Each of the mentioned processes or associated disease conditions are explained in the text (adapted from Ngan & Tam, 2008).

PK1 and PKR1 are more widely distributed in peripheral tissues such as reproductive, gastrointestinal, and blood systems than PK2 and PKR2, who are expressed more widely in the central nervous system (Michelle Y Cheng, Leslie, & Zhou, 2006). High levels of both PKR1 and PKR2 are seen in steroidogenic glands (ovary, testis, adrenal gland and placenta), and PKR2 knockout mice exhibit arrested spermatogenesis (Masumoto et al., 2006). Interestingly, in the fallopian tube, PKR1 expression is stimulated by nicotine exposure, via nicotinic AChRalpha-7 (Shaw et al., 2010).

In the brain, neurons express both PKR1 and PKR2, with PKR2 expression predominant in the brain overall. Yet in astrocytes, PKR1 expression is predominant, with
minimal expression of PKR2 (Puverel, Nakatani, Parras, & Soussi-Yanicostas, 2009). The non-selectivity of the PKs and differential expression of PKR1 vs PKR2 suggests that the effect of the ligands PK1 and PK2 are mediated depending on the receptor that are present at the target tissues. For instance, corpus luteum-derived endothelial cells (LEC) express both PKR1 and PKR2, whereas aorta endothelial cells (BAEC) and brain capillaries endothelial cells (BCEC) express only PKR1. In these tissue types, PK1 is mitogenic for LEC and BAEC endothelial cells, but has no effect on BCEC endothelial cells (Monnier & Samson, 2010).

PK2 is expressed in the bone marrow, and responds to granulocyte colony-stimulating factor for inducing hematopoietic cell proliferation (Shojaei et al., 2007). Importantly, PK2 expression can also be stimulated by hypoxia through HIF1α to induce growth and angiogenesis by endothelial cells. Similarly, PK2 can be activated by STAT3, which induces proliferative effects (Kujawski et al., 2008). Due to these mitogenic effects, PK2 is implicated in tumorigenesis and survival of tumors in hypoxic conditions. The PK2/PKR1 signaling axis promotes cardiomyocyte survival and angiogenesis, and in the mouse model of myocardial infarction, transient PKR1 transfection in the heart could protect the heart’s structure and function (Boulberdaa, Urayama, & Nebigil, 2011). Mechanistically, overexpressing PK2 or PKR1 activates AKT to protect cardiomyocytes against oxidative stress, and siRNA against PKR1 completely reversed the protective effects (Urayama et al., 2007). Further, loss of PKR1 in mice leads to heart and kidney abnormalities due to mitochondrial defects (Nebigil, 2010).

One of the earliest discovered properties of PK2 is its ability to induce hyperalgesia to pain stimulus when injected into paws of rats. The majority of dorsal root ganglion cells responding to stimuli co-express both PKR1 as well as the transient receptor potential vanilloid 1 (TRPV1), and PKR1 receptor activation potentiates the activation of TRPV1, significantly
lowering the nociceptor threshold to physical and chemical stimuli (Lucia Negri et al., 2002). PKR1-null mice showed impaired acute nociception, compromising what is an essentially protective response against further injury.

PK1 could induce differentiation of bone marrow cells into monocyte/macrophages (Dorsch et al., 2005), and is highly expressed in the endothelial cells of blood vessels and the ovaries to induce angiogenesis, giving it the alternative name endocrine gland vascular endothelial growth factor (EG-VEGF) (Fraser et al., 2005). A number of PK1, PKR1, PKR2 polymorphisms are associated with recurrent pregnancy loss (Y. Cao et al., 2016; M.-T. Su et al., 2010; M. T. Su, Lin, Chen, & Kuo, 2014; Mei-Tsz Su et al., 2010). These and other studies had found that during pregnancy, the expression of EG-VEGF/PK1 and PKR1, PKR2 are controlled temporally in human placenta of the first and third trimester period. This expression pattern suggests their important roles, especially during human early pregnancy, when vascularization of the chorionic villi in the placenta is essential for successful pregnancy. The dynamic expression of PK1 may be regulated by estrogen, progesterone and human chorionic gonadotrophin, and has also been proposed to be regulated by HIF-1α via hypoxia-response element in the promoter region of PK1. Several polymorphisms, PROKR1 (I379V) and PROKR2 (V331M) conferred protection in recurrent pregnancy loss via decreased intracellular calcium influx and increased cell invasiveness (M. T. Su, Lin, Chen, Wu, & Kuo, 2013). Cell proliferation, cell–cell adhesion, and tube organization are not affected, and the mechanisms of protection owing to the polymorphisms are not completely understood.
PK1-GDNF Signaling in the Enteric Nervous System

In the enteric nervous system, PK1 is indispensable for the development of enteric neurons. PK1, but not PK2, is expressed in mouse embryonic gut during enteric nervous system development (Ngan et al., 2007). During this critical period, dysregulation in GDNF or PK1 signaling in the enteric system causes defects in proliferation and differentiation of neural crest stem cells, and cause a condition in humans known as Hirschsprung disease (Iwashita, Kruger, Pardal, Kiel, & Morrison, 2003). Mechanistically, the proliferative and differentiation effects of PK1 is potentiated by GDNF via upregulating PKR1 expression in enteric neural crest stem cells. Further, overexpression of PKR1 could rescue a lack of RET signaling, suggesting that PK1 and GDNF signaling pathways share some common downstream targets (Ngan et al., 2007).

However, little is known about the involvement of prokineticin signaling with GDNF in the central nervous system during development, and even less during neurodegenerative processes. Since PK2, and not PK1, participates as key regulator of biological processes in the brain, the following sections will focus on the function of PK2 in the brain and during neurodegenerative processes.

PK2 Signaling in the Brain

PK2 transcriptional regulation by circadian rhythm

In the brain, PK2 and PKR2 are both highly expressed in the hypothalamus, the limbic system, and olfactory bulbs. PK2 is also highly expressed in the suprachiasmatic nucleus (SCN), the regulator of circadian rhythm in mammals. Levels of PK2 expression oscillates
from the highest during the day to almost undetectable at night, and is entrainable by light (Michelle Y. Cheng et al., 2002). In vitro studies revealed that PK2 is under the control of first-order clock-controlled genes, particularly the basic helix-loop-helix transcription factors (bHLH) CLOCK-BMAL1, which are master regulator of circadian rhythm. CLOCK-BMAL1 could transcriptionally regulate PK2 gene expression by binding to enhancer-box (E-Box) element sequence (CACGTG) in the PK2 proximal promoter region. Soon it was also found that other bHLH transcription factors Ngn1 and MASH1/ASCL1 could also bind to the PK2 promoter to regulate its effects on neurogenesis (C. Zhang et al., 2007), HIF1α, as a bHLH transcription factor, was also postulated to bind to the PK2 promoter.

In vivo studies indicated that mice lacking the clock gene also have drastically reduced oscillation of PK2 expression in the SCN. Moreover, intracerebroventricular injection of PK2 protein disrupts normal nocturnal locomotor activity in nocturnal mice. Interestingly, injection of PK2 into the arcuate nucleus, a target of SCN, could suppress feeding without affecting drinking. When injected into the subfornical organ, an indirect target of SCN via the paraventricular nuclei (PVN), PK2 stimulated drinking without affecting feeding (Lucia Negri et al., 2004). Therefore, PK2 output from the SCN could transmit the circadian rhythm of feeding and water regulation in the body. Studies in diurnal rats revealed similar temporal expression pattern as nocturnal mice, which seems to indicate that diurnality lies downstream of the SCN for rodents (Lambert, Machida, Smale, Nunez, & Weaver, 2005). However, PK2 antagonist could produce opposite effects of the PK2 signaling on the arousal levels in the nocturnal mice and diurnal monkey, which could be attributable to the differential expression of receptors for PK2 in the intrinsically photosensitive retinal ganglion cells (ipRGC) that transmit photonic information to SCN for the two animals, therefore suggesting that diurnality
could lie upstream of the SCN starting from the response of ipRGC to light (Qun Yong Zhou et al., 2016).

**PK2 induces neurogenesis**

PK2 signaling is necessary for olfactory bulb neurogenesis and the continuing migration of neuroprogenitor cells (NPC) from the subventricular zone (SVZ) through the rostral migratory stream (RMS) during adulthood. PK2 is highly expressed in the olfactory bulbs, where it acts as a chemoattractant for SVZ-born neuroprogenitor cells migrating towards the olfactory bulb (Ng et al., 2005). In the SVZ, accumulation of pkr2 transcripts was detected in almost all migrating neuroblasts (Puverel et al., 2009). In the absence of PK2 signaling, it seems that the neuroprogenitors cells do not detach from the rostral migratory stream properly, or they are disoriented about the direction of migration (Winner & Winkler, 2015). In Pk2−/− mice, the olfactory bulbs do not develop normally and are less than half the size of wildtype controls, and exhibit multiple abnormalities in the various layers of the olfactory bulbs (Ng et al., 2005). Interestingly, during early development, the lack of organization of the olfactory bulb hinders the migration of a group of neurons, gonadotrophin-releasing hormone (GnRH) neurons, from the olfactory placode in the peripheries, through the olfactory bulb, to the hypothalamus, where they extend processes to the median eminence. The precise mechanisms that guide these small group of neurons through such long distances are unclear, but nonetheless, the lack of organization of the olfactory bulb obfuscates their migratory path. The failure of this group of neurons to migrate in turn results in a lack of gonadotrophin-releasing hormone normally secreted during puberty (Schwarting, Wierman, & Tobet, 2007). Thus, due to its crucial involvement in the morphogenesis of the mature olfactory bulb, loss of PK2
signaling arising from defects in either PK2 or PKR2 in humans causes a severe form of Kallmann syndrome, characterized by both anosmia and lack of puberty (S.-H. Kim, 2015). Similar to the case with PKR2 involvement in pregnancy, PKR2 polymorphisms could strongly influence disease severity. In three disease-associated mutations of the PKR2, W178S, G234D, and P290S, the mutant receptors are trapped in the cellular secretory pathway within the cell, never transported to the cell surface or properly integrated into the cell membrane (D. N. Chen, Ma, Liu, Zhou, & Li, 2014).

**PKR1/PKR2 Agonists and Antagonists**

While PK1 and PK2 make are endogenous and convenient agonists to the cognate PKR1 and PKR2 receptors, efforts have been made to synthesize smaller or more efficient agonists, and to look for antagonists of the receptors. Early studies had found that changes in the conserved N-terminal sequences result in the loss of agonist activity. The first antagonists were made such a way – substitution of alanine with methionine at position 1 created A1MPK1, and addition of a methionine to the N terminus created MetPK1 (Bullock et al., 2004).

A chemical Prokineticin receptor antagonist, 6-[(2-Amino-pyridin-3-ylmethyl)-amino]-1,3-bis-(4-methoxy-benzyl)-1H-[1,3,5] triazine-2,4-dione, was able to block PK2-induced increases in intracellular Ca^{2+} mobilization in CHO cells transfected with human PKR1 or PKR2 (Watson et al., 2012). No off-target effects were detected in a Novartis safety panel of 60 different receptors, suggesting a high-selectivity for prokineticin receptors.

From patented chemical structures release by what was known as Janssen Pharmaceuticals, 3 related compounds, with general structure 1,3,5-triazin-4,6-diones, were synthesized and were shown to inhibit Bv8/PK2-induced intracellular Ca^{2+} mobilization
(Balboni et al., 2008). The most effective compound, triazine Compound 1, at 300 nM, was able to inhibit close to 96% of cells from responding to 1 nM of Bv8/PK2-induced intracellular Ca^{2+} mobilization, suggesting that these triazine compounds are potential pharmacological prokineticin receptor antagonists (Balboni et al., 2008).

Recently, three more compounds, PKR-A, PKRA7, and A547 were synthesized and found to have antagonistic activities against either or both of the receptors. PKRA7, a PKR2-prefering antagonist, has been found to have anti-tumor activity in glioblastoma and pancreatic cancer xenograft tumor models (Curtis et al., 2013). PKR-A is a receptor antagonist that blocks both PKR1 and PKR2. It belongs to a group of morpholine carboxamide prokineticin antagonists (Patent US7855201), and was found to inhibit PKR2 at a IC_{50} of 48.1 ± 4.6 nM, in CHO cells stably expressing PKR2 (M Y Cheng et al., 2012). An interesting case lies with A457, another morpholine carboxamide prokineticin antagonist. As previously mentioned, three disease-associated mutations of the PKR2 (W178S, G234D, and P290S), cause deficiency in trafficking, resulting in retention of PKR2 within the cellular secretory pathways (D. N. Chen et al., 2014). Using a different modeling methodology employing the use of Phyre2 (Protein Homology/Analog Y Recognition Engine), the PKR2 W178S, G234D, and P290S sequences were modelled. Interestingly, A457 dramatically increased cell surface expression and rescued the function of PKR2 with P290S mutation in a dose- and time-dependent manner, without de novo protein synthesis. Data showed that A457 could affect the conformation only around its binding site. It is therefore thought that P290S mutation causes a subtle distortion in transmembrane domain VI, and binding of A457 allosterically to the mutant receptor enables it to act as a pharmacological chaperone, to affect its conformation and correct the misfolding (D. N. Chen et al., 2014).
In the recent few years, the first PKR1 agonists were designed, synthesized, and characterized. A group based in France used a homology model computational screening method to screen a library of 250,000 compounds and found 10 potential hits, which were tested \textit{in vitro} (Gasser et al., 2015). One compound was found to mobilize intracellular Ca\textsuperscript{2+}, and activate AKT, ERK signaling. Thirty more compounds, named IS1 through IS30, were then synthesized based on this parent compound. The most potent derivative, IS20, was confirmed for its selectivity and specificity to PKR1. Using this compound, the group showed that IS20 prevented cardiac lesion formation after myocardial infarction, and improved cardiac function. In line with numerous previously published \textit{in vitro} studies using endogenous PKR1 agonists which confirmed PKR1’s proliferative effects in cardiomyocytes (Boulberdaa, Turkeri, et al., 2011; Boulberdaa, Urayama, et al., 2011; Nebigil, 2010; Urayama et al., 2007), IS20 was shown to promote proliferation of cardiac progenitor cells and neovasculogenesis in the heart (Gasser et al., 2015).

We previously showed that PK2 mRNA was highly induced by TNF\textalpha in dopaminergic cell culture, and by neurodegeneration in animal models of PD. In MPP\textsuperscript{+} and MPTP classic Parkinsonian toxicant models, upregulation of PK2 in dopaminergic cells rescued MPP\textsuperscript{+}-induced cell death in cell culture and gene delivery of PK2 rescued MPTP-induced cell death \textit{in vivo} [6]. Our data therefore supported the hypothesis that the upregulation of PK2 is a neuroprotective compensatory response relevant in PD-related neurodegeneration. Given the availability of PKR1 agonists, we aim to evaluate the therapeutic potential of PKR1 agonist in activating protective prokineticin signaling in animal model of PD. Clearly, prokineticin signaling is a complex network involving multiple modes of signal transduction in various cell
types, and it is our goal to further elucidate its mechanism of action, especially during neurodegenerative stress events.
CHAPTER 2. TRANSCRIPTIONAL REGULATION OF PROKINETICIN-2 PROMOTER BY EGR1, HIF1A, AND NRF1 IN CELL CULTURES OF DOPAMINERGIC NEURODEGENERATION

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Abstract

While cell signaling mechanisms underlying neurotoxic injury have been actively studied in recent years, signaling molecules contributing to compensatory survival signaling are largely unknown. Recently, we reported that the secreted neuropeptide prokineticin-2 (PK2) is upregulated during early stages of neurotoxic stress induced by MPP⁺, and plays a major compensatory protective function in nigral dopaminergic neurons in vitro and in vivo. To further study the transcriptional and molecular regulatory mechanisms of MPP⁺ and Mn-induced PK2 upregulation, we performed in silico analysis of the PK2 promoter and revealed the presence of (early growth response-1) EGR1, HIF1α (hypoxia inducible factor 1), transcription factor E2F, and nuclear respiratory factor-1 (NRF1) putative binding sites. Importantly, we observed that MPP⁺ and Mn exposure increased HIF1α and EGR1 levels at early stages of neurotoxicity. Since overexpression of HIF1α or EGR1 upregulated PK2 expression, these studies suggest that Mn regulates PK2 expression via EGR1 or HIF1α-dependent pathway. A toxicologically relevant dose of Mn exposure by an oral route (30 mg/kg
for 30 days) significantly upregulated global PK2 levels in the brain, especially in the substantia nigra with concomitant increases in HIF1α transcription factor. Taken together, these results suggest that Mn upregulates PK2 levels to counter the early neurotoxic stress and that Mn-induced upregulation of PK2 expression is transcriptionally regulated by EGR1, HIF1α and NRF1.

**Introduction**

Parkinson’s disease (PD) is characterized by a progressive degeneration of dopaminergic neurons in the substantia nigra in the ventral midbrain, resulting in reduction of dopamine in the striatum and ensuing chronic deficits in motor functions (Kalia & Lang, 2015; Poewe, 2010). Clinically, nonmotor symptoms also exist, and manifests itself through olfactory deficits, depression, and constipation (Poewe, 2010). Following the Nobel-winning discovery of the role of dopamine precursor 3,4-dihydroxy-L-phenylalanine (L-dopa) in PD by the Swedish scientist Arvid Carlsson in the 1950’s (W & O, 1961), the standard-of-care for PD have continued to be the administration of L-DOPA for the management of motor symptoms, while having little impact on the progressively worsening neurodegeneration associated with the condition. In addition, symptoms such as difficulties with balance, swallowing, or nonmotor symptoms such as speech or cognition can be progressively resistant or unresponsive to L-DOPA therapy (Okun, 2012).

Biochemically, the involvement of mitochondrial dysfunction have been recognized as a major contributor to disease pathology and progression (Ghosh et al., 2010; Gómez-Sánchez, Bravo-San Pedro, Gegg, González-Polo, & Fuentes, 2016; Jin et al., 2014; Keane, Kurzawa, Blain, & Morris, 2011; Mounsey & Teismann, 2010; Schapira & Gegg, 2011). Given that mitochondrial dysfunction has been increasing recognized as a central theme in other
neurodegenerative disease such as in Alzheimer’s and ALS (Cozzolino, Ferri, Valle, & Carri, 2013; Maruszak & Żekanowski, 2011; Von Lewinski & Keller, 2005; Yu et al., 2018), pathways that stimulate mitochondrial biogenesis represent druggable targets against neurodegeneration.

Mn can accumulate in the brain, a major organ of Mn toxicity during overexposure. The disruption of the nigrostriatal pathway of the basal ganglia system in prolonged Mn overexposure causes a severe neurological disorder similar to Parkinson’s disease named Manganism (Cawte, Hams, & Kilburn, 1987). Manganism is characterized clinically by extrapyramidal symptoms resembling Parkinson’s disease, with depression, postural instability, bradykinesia, micrographia, and a characteristic cock-walk caused by dystonia of the legs (Cersosimo & Koller, 2006; Olanow, 2004; Perl & Olanow, 2007).

The involvement of oxidative stress has also been recognized as a major contributor to Parkinsonism progression. In the substantia nigra, the pro-inflammatory tumor necrosis factor alpha (TNFα) could induce both protein kinase C delta (PKCδ)-mediated upregulation of reactive oxygen species (ROS), and microglial NADPH oxidase-mediated free radicals generation (Gordon, Singh, et al., 2016; Langley et al., 2018). Furthermore, ROS could prevent degradation of hypoxia-inducible factor-1alpha (HIF1α) under normoxic conditions (Park et al., 2003), and DHB, a HIF1α stabilizer, was shown to be neuroprotective in a MPTP model of PD (D. W. Lee et al., 2009), suggesting that HIF1α could naturally be a compensatory mechanism for neurons under hypoxic stress as well as oxidative stress. Studies in choroidal neovascularization (CNV) indicated that HIF1α expression is regulated by AKT phosphorylation (Yang et al., 2009), while in in cell cultures of PD, phosphorylation and activation of PI3K/AKT pathway could rescue neurodegeneration of dopaminergic neurons.
during neurotoxic stress, which stimulates neurotrophic factor upregulation and counters neurodegeneration by inhibition of pro-apoptotic signals. Therefore, although much focus had been on deciphering the process of neuronal cell death, increasing efforts have more recently been placed on identifying early compensatory responses to neurodegeneration.

Prokineticin-2 (PK2) is a signaling neuropeptide that regulates wide-ranging functions as gut motility, angiogenesis hematopoiesis, estrous cycle progression, and nociception (Hu et al., 2006; Negri & Lattanzi, 2012; Negri, Lattanzi, Giannini, & Melchiorri, 2007; Ngan & Tam, 2008; Soderhall, Kim, Jiravanichpaisal, Lee, & Soderhall, 2005; Xiao et al., 2014). In the central nervous system (CNS), PK2 could also participate in neurogenesis (Ng et al., 2005). Furthermore, PK2 expression can be transactivated via basic helix-loop-helix (bHLH) transcription factors binding to several Enhancer-box (E-box) elements in its proximal promoter region. Bmal, a bHLH transcription factor part of the core component of circadian rhythm pacemaker, targets PK2 as one of its approximately 150 sites (Hatanaka et al., 2010), and activates PK2 gene expression via binding to E-box elements in its promoter as a heterodimer in a cyclic, rhythmic manner, resulting in its secretion from the suprachiasmatic nucleus (Cheng et al., 2002; J. Da Li, Hu, & Zhou, 2012; Menet, Pescatore, & Rosbash, 2014; Zhang et al., 2007; Q Y Zhou, 2006; Qun Yong Zhou & Cheng, 2005).

The mitogenic effects of PK2 in the peripheries have been well characterized (Keramidas, Faudot, Cibiel, Feige, & Thomas, 2008; Kislouk et al., 2005; LeCouter et al., 2003; LeCouter, Zlot, Tejada, Peale, & Ferrara, 2004; Podlovni et al., 2006), but its role in CNS have been less well studied (Koyama et al., 2006; Ng et al., 2005), especially during neurodegeneration. During an initial screening of early gene expression changes in responses to neuroinflammation, we had previously discovered that PK2 became highly upregulated in
response to pro-inflammatory factors, which upon subsequent examination in PD animal models, were upregulated in nigral dopaminergic neurons preceding the onset of motor deficits and participated in an early, protective compensatory response to neurodegeneration (Gordon, Neal, et al., 2016). Mechanistic studies later showed that PK2 signaling protected against the Parkinsonian neurotoxicant MPP⁺ by countering oxidative stress, mitochondrial dysfunction and dopaminergic degeneration. However, the upstream mechanisms that induces PK2 upregulation during neurodegeneration remains incompletely understood, and our goal is to integrate PK2’s role into the current understanding of compensatory, pro-survival signaling networks during neurodegeneration.

During early stages of neurotoxicity, compensatory responses that counter pro-apoptotic signals, stimulate mitochondrial biogenesis, are likely governed by complex networks of transcription factors. We hypothesized that PK2 gene expression is regulated by a number of such transcription factors which are known to associate with pro-survival responses and mitochondrial biogenesis. In this study, we examined the regulation of PK2 gene by the toxicant-responsive transcription factor EGR1, mitochondrial regulator NRF1, as well as pro-survival factor HIF1α during response to Parkinsonian toxicant MPP⁺ and Mn. We found that during neurotoxic stress, EGR1, NRF1, and HIF1α could positively regulate PK2 gene transcription possibly by binding to putative EGR1, NRF1, or HIF1α binding sites, respectively. The expression of the PK2 is reduced by disruption of EGR1 or HIF1α signaling in dopaminergic neurons. Our results indicate that PK2 is a major downstream target of EGR1, NRF1, and HIF1α transcription factors during neurotoxic stress.
Results

We had previously shown that PK2 upregulation is a protective, compensatory response to neurodegeneration, and over expression could induce mitochondrial biogenesis (Gordon, Neal, et al., 2016). Here, we seek to elucidate PK2’s transcriptional regulation during neurotoxic stress.

**Figure 1. Neurotoxicant MPP+ upregulates PK2 and induces PK2 promoter activity in dopaminergic neurons.** Parkinsonian toxicant MPP+ has a EC$_{50}$ of roughly 300µM during a 24h treatment in cultured dopaminergic neurons, and induces significant caspase activation at 8h (Gordon, Neal, et al., 2016). Quantitative PCR assay showed that Parkinsonian toxicants MPP+, Mn, as well as HIF1α stabilizer DHB, could induce mRNA expression in MN9D cell (Figure 1A). Quantitive PCR assay also showed that MPP+ treatment at 300µM in MN9D cultured mouse dopaminergic neurons upregulates PK2 gene expression during early neurotoxic stress, peaking at 3 hours post treatment, an early event that precedes dopaminergic cell death (Figure 1B). Western blot assay similarly showed early increases in PK2 protein level at 3h that remained high at 12h (Figure 1C). This suggests that PK2 is an early responder to MPP+ neurotoxicity. However, it is unclear if PK2 upregulation was only due to mRNA stabilization and posttranslational modifications on mature protein, or if PK2 expression was also regulated by transcriptional control during neurotoxic stress. Therefore, to determine if MPP+ could induce PK2 transcriptional activation, we cloned a 1kb fragment of the proximal promoter immediately upstream of the human PK2 gene putative transcription start site into a promoterless Nanoluc vector plasmid construct (pNL) encoding a nanoluciferase reporter. We found that MPP+ increased PK2 promoter activity in MN9D dopaminergic cell host transfected with the PK2 promoter construct, increasing luciferase activity at 3h and 6h-post treatment by 2-fold, which indicates that PK2 response to MPP+ can be controlled at the level of
transcription (Figure 1D). These experiments indicate that neurotoxicant such as MPP+ can increase level of PK2 by inducing its transcription.

**Figure 2. In silico analyses reveal conserved, methylated sequences 1kb upstream of proximal PK2 promoter, and a wide range of transcription factors associated with metabolic processes, cellular processes, cellular biogenesis, and transcription factors regulating response to stimuli.** PK2 has been shown to participate in a compensatory, protective response during neurotoxic stress against neurodegeneration (Gordon, Neal, et al., 2016). While the downstream effects of PK2 on MAPK/ERK signaling has been elucidated, little is known about the upstream events and mechanisms that lead to PK2 upregulation during neurotoxicity. Previous studies had found that positive regulation of PK2 gene could be achieved by transcriptional factor binding to E-Box, which are present in multiple copies within 1kb upstream of the transcription start site (Cheng et al., 2002; Cheng, Bittman, Hattar, & Zhou, 2005; J.-D. Li et al., 2006; Qun Yong Zhou & Cheng, 2005). Since induction of PK2 transcription during neurotoxic stress cannot be expected to be wholly elicited by circadian rhythm-responsive genes, we sought to analyze the proximal PK2 promoter region and comprehensively predict putative transcription factor binding sites. Firstly, to determine a putative promoter region, we utilized the ECR browser (ecrbrowser.dcode.org), to compare human PK2 promoter sequences with other species, which revealed that 1kb upstream of the transcription start site is conserved across rodents and canines (Figure 2A). Analysis of promoter CpG islands using EMBOSS CpGplot found increased CpG around -400 to +1 relative to transcription start site (Figure 2B), and MethPrimer analysis (L. C. Li & Dahiya, 2002) found two CpG islands near -400 to +1 of the transcription start site (Figure 2C). These software analyses for appearance of CpG suggest the presence of a core promoter region
(Deaton & Bird, 2011; Saxonov, Berg, & Brutlag, 2006). To comprehensively predict putative transcription factor binding sites, the 1kb upstream promoter sequences was uploaded into the MatInspector software (Cartharius et al., 2005; Quandt, Frech, Karas, Wingender, & Werner, 1995) from Genomatix (Figure 2D). Using Matrix Similarity cutoff > 0.66, (indicating high confidence of putative transcription factor binding site), MatInspector database and algorithms identified 77 transcription factors that could bind to 1kb upstream proximal promoter for the PK2 gene (Table 1).

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZF04</td>
<td>C2H2 zinc finger transcription factors 4</td>
</tr>
<tr>
<td>DEAF</td>
<td>Homolog to deformed epidermal autoregulatory factor-1 from D. melanogaster</td>
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<tr>
<td>HDBP</td>
<td>Huntington's disease gene regulatory region binding proteins</td>
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<tr>
<td>RU49</td>
<td>Zinc finger transcription factor RU49, zinc finger proliferation 1 - Zipro1</td>
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<td>PAX1</td>
<td>PAX-1 binding sites</td>
</tr>
<tr>
<td>AHRR</td>
<td>AHR-ant heterodimers and AHR-related factors</td>
</tr>
<tr>
<td>NF1F</td>
<td>Nuclear factor 1</td>
</tr>
<tr>
<td>CTCF</td>
<td>CTCF and BORIS gene family, transcriptional regulators with 11 highly conserved zinc finger domains</td>
</tr>
<tr>
<td>ZICF</td>
<td>Members of ZIC-family, zinc finger protein of the cerebellum</td>
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<tr>
<td>PLAG</td>
<td>Pleomorphic adenoma gene</td>
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<tr>
<td>ZF5F</td>
<td>ZF5 POZ domain zinc finger</td>
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<tr>
<td>HAND</td>
<td>Twist subfamily of class B bHLH transcription factors</td>
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<tr>
<td>RXRF</td>
<td>RXR heterodimer binding sites</td>
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<tr>
<td>API1</td>
<td>MAF and AP1 related factors</td>
</tr>
<tr>
<td>HOMF</td>
<td>Homeodomain transcription factors</td>
</tr>
<tr>
<td>SORY</td>
<td>SOX/SRY-sex/testis determining and related HMG box factors</td>
</tr>
<tr>
<td>KLFS</td>
<td>Krueppel like transcription factors</td>
</tr>
<tr>
<td>BNCF</td>
<td>Basonuclein rDNA transcription factor (PolI)</td>
</tr>
<tr>
<td>GCMF</td>
<td>Chorion-specific transcription factors with a GCM DNA binding domain</td>
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<td>RORA</td>
<td>v-ERB and RAR-related orphan receptor alpha</td>
</tr>
<tr>
<td>SPZ1</td>
<td>Testis-specific bHLH-Zip transcription factors</td>
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<td>Table 1. Continued</td>
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<tr>
<td><strong>EGRF</strong></td>
<td>EGR/nerve growth factor induced protein C &amp; related factors</td>
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<tr>
<td><strong>ZF02</strong></td>
<td>C2H2 zinc finger transcription factors 2</td>
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<tr>
<td><strong>SPIF</strong></td>
<td>GC-Box factors SP1/GC</td>
</tr>
<tr>
<td><strong>RREB</strong></td>
<td>Ras-responsive element binding protein</td>
</tr>
<tr>
<td><strong>GLIF</strong></td>
<td>GLI zinc finger family</td>
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<tr>
<td><strong>E2FF</strong></td>
<td>E2F-myc activator/cell cycle regulator</td>
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<tr>
<td><strong>MAZF</strong></td>
<td>Myc associated zinc fingers</td>
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<tr>
<td><strong>SRFF</strong></td>
<td>Serum response element binding factor</td>
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<td><strong>HIF</strong></td>
<td>Hypoxia inducible factor, bHLH/PAS protein family</td>
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<td><strong>FKHD</strong></td>
<td>Fork head domain factors</td>
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<tr>
<td><strong>NEUR</strong></td>
<td>NeuroD, Beta2, HLH domain</td>
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<td><strong>RP58</strong></td>
<td>RP58 (ZFP238) zinc finger protein</td>
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<tr>
<td><strong>MYOD</strong></td>
<td>Myoblast determining factors</td>
</tr>
<tr>
<td><strong>HNF1</strong></td>
<td>Hepatic Nuclear Factor 1</td>
</tr>
<tr>
<td><strong>PBXC</strong></td>
<td>PBX1 - MEIS1 complexes</td>
</tr>
<tr>
<td><strong>43374</strong></td>
<td>Octamer binding protein</td>
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<tr>
<td><strong>MYT1</strong></td>
<td>MYT1 C2HC zinc finger protein</td>
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<tr>
<td><strong>EBOX</strong></td>
<td>E-box binding factors</td>
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<tr>
<td><strong>HES</strong></td>
<td>Vertebrate homologues of enhancer of split complex</td>
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<td><strong>CREB</strong></td>
<td>cAMP-responsive element binding proteins</td>
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<td><strong>ZF08</strong></td>
<td>C2H2 zinc finger transcription factors 8</td>
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<td><strong>NR2F</strong></td>
<td>Nuclear receptor subfamily 2 factors</td>
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<tr>
<td><strong>XBBF</strong></td>
<td>X-box binding factors</td>
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<tr>
<td><strong>NFAT</strong></td>
<td>Nuclear factor of activated T-cells</td>
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<tr>
<td><strong>CAAT</strong></td>
<td>CCAAT binding factors</td>
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<td><strong>NGRE</strong></td>
<td>&quot;Negative&quot; glucocorticoid response elements</td>
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<td><strong>NRSF</strong></td>
<td>Neuron-restrictive silencer factor</td>
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<td><strong>ZF06</strong></td>
<td>C2H2 zinc finger transcription factors 6</td>
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<td><strong>GRHL</strong></td>
<td>Grainyhead-like transcription factors</td>
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<tr>
<td><strong>ETSF</strong></td>
<td>Human and murine ETS1 factors</td>
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<tr>
<td><strong>NRF1</strong></td>
<td>Nuclear respiratory factor 1</td>
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<tr>
<td><strong>RBPF</strong></td>
<td>RBPJ - kappa</td>
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<tr>
<td><strong>BARB</strong></td>
<td>Barbiturate-inducible element box from pro+eukaryotic genes</td>
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<tr>
<td><strong>MYBL</strong></td>
<td>Cellular and viral myb-like transcriptional regulators</td>
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<td><strong>MOKF</strong></td>
<td>Mouse Krueppel like factor</td>
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<td><strong>MTEN</strong></td>
<td>Core promoter motif ten elements</td>
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<td><strong>AP2F</strong></td>
<td>Activator protein 2</td>
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<tr>
<td><strong>TF2B</strong></td>
<td>RNA polymerase II transcription factor II B</td>
</tr>
<tr>
<td><strong>CHRE</strong></td>
<td>Carbohydrate response elements, consist of two E box motifs separated by 5 bp</td>
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<tr>
<td><strong>HASF</strong></td>
<td>HIF-1 ancillary sequence family</td>
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<tr>
<td><strong>SMAD</strong></td>
<td>Vertebrate SMAD family of transcription factors</td>
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<tr>
<td><strong>SREB</strong></td>
<td>Sterol regulatory element binding proteins</td>
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Table 1. Continued

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<tr>
<th>Gene</th>
<th>Description</th>
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<tr>
<td>OAZF</td>
<td>Olfactory associated zinc finger protein</td>
</tr>
<tr>
<td>XCPE</td>
<td>Activator-, mediator- and TBP-dependent core promoter element for RNA polymerase II transcription from TATA-less promoters</td>
</tr>
<tr>
<td>AP1F</td>
<td>AP1, Activating protein 1</td>
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<tr>
<td>P53F</td>
<td>p53 tumor suppressor</td>
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<tr>
<td>VTBP</td>
<td>Vertebrate TATA binding protein factor</td>
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<tr>
<td>CDXF</td>
<td>Vertebrate caudal related homeodomain protein</td>
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<tr>
<td>OVOL</td>
<td>OVO homolog-like transcription factors</td>
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<tr>
<td>NOLF</td>
<td>Neuron-specific olfactory factor</td>
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<tr>
<td>CP2F</td>
<td>CP2-erythrocyte Factor related to drosophila Elf1</td>
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<td>PAX3</td>
<td>PAX-3 binding sites</td>
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<td>AP4R</td>
<td>AP4 and related proteins</td>
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<tr>
<td>MTF1</td>
<td>Metal induced transcription factor</td>
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<tr>
<td>PAX9</td>
<td>PAX-9 binding sites</td>
</tr>
<tr>
<td>WHNF</td>
<td>Winged helix binding sites</td>
</tr>
</tbody>
</table>

The PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System for Gene Ontology (Mi, Muruganujan, & Thomas, 2013; Thomas et al., 2003), can be used to classify gene functions into broad categories (Mi, Muruganujan, Casagrande, & Thomas, 2013; PANTHER, 2016). We found that among the putative transcription factors that regulate biological processes, transcription factors that regulate metabolic processes represents 25% of all transcription factors and cellular processes represented 25% of transcription factors, two of the most enriched ontology terms (Figure 2E). Developmental processes represented 10.7%, cellular component organization or biogenesis represented 7.1%. Transcription factors that regulate response to stimuli represented 10.7%, biological regulation represented 7.1%, and multicellular organismal process represented 22.2%. Number of genes that correspond to each category is listed as a histogram (Figure 2F). However, not all putative transcription factors were predicted to express in the CNS. Therefore, to narrow our focus and avoid false positives, we filtered the names of predicted transcription factors.
factors using MatInspector’s database to search for known expression in a specific tissue organ. When filters for specific expression in CNS was applied, 23 transcription factors remained, and they were classified according to function (Table 2).

Table 2. Putative PK2 proximal promoter binding-transcription factors that are known to express in the CNS.

| Regulation of proliferation and differentiation | | |
|----------------------------------|----------------------------------|
| EGFR | Early Growth Response/nerve growth factor induced protein C |
| GLIF | GLI zinc finger family |
| HASF | HIF-1 ancillary sequence family |
| HIFF | Hypoxia inducible factor, bHLH/PAS protein family |
| MYT | MYT1 C2HC zinc finger protein |
| NRSF | Neuron-restrictive silencer factor |
| PLAG | Pleomorphic adenoma gene |
| RP58 | RP58 (ZFP238) zinc finger protein |
| BRN | Brn-5 POU domain factors |
| GRHL | Grainyhead-like transcription factors |
| NRF1 | Nuclear respiratory factor 1 |

| Cell development and maintenance | | |
|----------------------------------|----------------------------------|
| CLOX | CLOX and CLOX homology (CDP) factors |
| E2F | E2F-myc activator/cell cycle regulator |
| HESF | Vertebrate homologues of enhancer of split complex |
| IRX | Iroquois homeobox transcription factors |
| SORY | SOX/SRY-sex/testis determining and related HMG box factors |

| Regulator of cell survival | | |
|---------------------------|----------------------------------|
| P53 | p53 tumor suppressor |
| DEAF | Homolog to deformed epidermal autoregulatory factor-1 |
| MOKF | Mouse Krueppel like factor |
| CTCF | CTCF and BORIS gene family, transcriptional regulators |

| Regulation of circadian rhythm | | |
|---------------------------------|----------------------------------|
| EBOX | E-box binding factors |
| RORA | v-ERB and RAR-related orphan receptor alpha |

| Regulation of metal homeostasis | | |
|----------------------------------|----------------------------------|
| MTF1 | Metal induced transcription factor |

The PANTHER classification for gene ontology was used again to analyze the resultant genes. Of the putative transcription factors binding to the PK2 proximal promoter that are
known to express in the CNS, transcription factors associated with cell process represented 23.8% and metabolic process represented 23.8%, again two of the most enriched gene ontology. Biological regulation represented 9.5% of transcription factors, cellular component organization or biogenesis represented 4.8%, developmental process represented 14.3%, multicellular organismal process represented 9.5%, and transcription factors that regulate response to stimulus represented 14.3% of transcription factors (Figure 2G, H).

The in silico analyses collectively suggested that transcription factors involved with cellular processes and metabolic processes are the most over-represented gene ontology terms. This is in line with currently known roles for PK2, such as cell process of cell-cell communication, and metabolic process resulting in cellular growth (Q. Y. Zhou & Meidan, 2008). We had shown previously that PK2 is responsive to neurotoxic stress, and at early stages of toxicity, it acts to compensate for the pro-apoptotic signals by inducing anti-apoptotic BCL-2 and mitochondrial biogenesis. Interestingly, regulation of stimulus and biogenesis are also represented in the unbiased analysis of transcription factors, possibly providing mechanisms for regulation of PK2 during neurotoxic stress. The unbiased analyses provided a starting point for further analysis and validation.

**Figure 3. PK2 may be a target gene for Early Growth Response family of transcription factors (EGRs), Nuclear Respiratory Factor 1 (NRF1), Hypoxia-inducible factor1 (HIF1α), and E2F-myc activator/cell cycle regulator.** Putative transcription factors that regulate cell proliferation/differentiation, or cell development/maintenance are over-represented in Table 2, suggesting that PK2, as a downstream target of these transcription factors, may be highly involved in the corresponding cellular functions. PK2 may be downstream target of proliferation-associated Early Growth Response family of transcription
factors (EGRs), mitochondrial biogenesis-inducing Nuclear respiratory factor 1 (NRF1), pro-survival Hypoxia-inducible factor alpha (HIF1α), and E2F-myc activator/cell cycle regulator (E2F).

The bHLH transcription factors MASH1 and Ngn1 were reported to positively regulate PK2 gene expression by binding to E-box elements in the proximal promoter, and HIF1α was speculated to interact with the PK2 promoter via similar mechanisms (Zhang et al., 2007; Q Y Zhou, 2006). Additional putative HIF1α binding sites in the proximal promoter revealed by in silico analysis in the present study further might suggest a role of HIF1α in PK2 gene regulation.

The corresponding conserved core binding sites on 1kb PK2 promoter is shown in Figure 3A. To test if PK2 upregulation during neurotoxic stress is modulated through EGRs, NRF1, or HIF1α, we treated MN9D dopaminergic neurons with Parkinsonian toxicant MPP+ or manganese, as well as oxidative stress-inducing hydrogen peroxide (Cantu, Schaack, & Patel, 2009). Western blot analysis showed that MPP+, Mn, or hydrogen peroxide drastically increased EGR1 protein level within 6h post-treatment, suggesting that EGR1 is responsive to neurotoxic stress (Figure 3B). PK2 mRNA was rapidly induced by MPP+ within 30 minutes and is sustained at 6h (Figure 3C). These studies suggest that EGR1, NRF1, E2F and HIF1α co-regulate with PK2 expression during a mitochondrial inhibition-induced neurotoxicity and oxidative stress-induced neurotoxicity.

Figure 4. Overexpression of EGRs, NRF1, HIF1α, or E2F1 in MN9D dopaminergic neurons induces PK2 promoter activity. Cotransfection of 1kb PK2 proximal promoter nanoluciferase reporter construct (3ug) with plasmid vector encoding wildtype EGR1, EGR2, EGR3, NRF1, HIF1α, HIF2α, E2F, or empty vector (9ug) for a total of 12ug
total transfected vector DNA. Promoter assay analyzing the expression and activity of nanoluciferase driven by the PK2 1kb proximal promoter indicated that members of the EGR family could stimulate PK2 promoter activity (Figure 4A). Similarly, NRF1 and HIF1α, also stimulated promoter activity (Figure 4B, 4C). Since the α subunit of HIF1 (HIF1α) is tightly controlled by prolyl hydroxylase, which under normoxic conditions, prevents its accumulation (Tennant et al., 2009), we used a prolyl hydroxylase inhibitor, DHB (ethyl 3,4 - dihydroxybenzoic acid) to stabilize HIF1α protein (Lomb, Straub, & Freeman, 2007; Siddiq et al., 2005) in MN9D dopaminergic neurons. While DHB treatment alone induced a moderate, 50% increase in promoter activity compared to mock treatment, DHB treatment in combination with HIF1α overexpression produced more than 200% increase in promoter activity (Figure 4C). Additionally, mutant HIF1α with defective PAS domain did not stimulate the PK2 promoter activity (Figure 4C). Since HIF2 is also needed for dopaminergic neuron survival in the SNpc(Smeyne, Sladen, Jiao, Dragatsis, & Smeyne, 2015), and some studies suggest that HIF2 might regulate prolonged hypoxic gene activation(Holmquist-Mengelbier et al., 2006), therefore we also tested and confirmed that HIF2 could upregulate PK2 promoter activity (Figure 4D). Overexpression of E2F drastically stimulated PK2 promoter activity, with nearly 3500% increase (Figure 4E). MatInspector analysis also revealed that two EGR1 binding sites overlapped with NRF1 binding sites (-64/-60, and -131/-129), and it could be speculated that EGR1 and NRF1 could synergistically stimulate PK2 gene expression. Therefore, in a separate study, we co-transfected PK2 promoter construct together with both EGR1 and NRF1 plasmid vectors. The amount of EGR1 and NRF1 plasmid vector DNA were reduced in half (4.5ug), so the total amount of transfected plasmid DNA is consistent across the study (12ug total). As controls, we transfected the same amount of EGR1 or NRF1 (4.5ug) for this experiment (for a
total of 7.5ug). As expected, we found increased promoter activity with EGR1 or NRF1-transfected cells compared to empty vector control.

To further confirm if overexpression of EGR1, NRF1, HIF1α or E2F1 could induce PK2 gene expression, MN9D dopaminergic neurons were separately transfected with plasmid DNA encoding cDNAs of these transcription factors for 16h, to allow for expression of plasmid DNA. Analysis using qPCR showed that EGR1 overexpression induced PK2 gene expression approximately 4.5-fold of control, while NRF1 overexpression induced > 3-fold of control, HIF1α overexpression induced > 4-fold of control, and E2F overexpression induced > 5-fold of control (Figure 4F-4I). Collectively, this set of experiments indicate that EGRs, NRF1, HIF1α, HIF2α, or E2F could induce PK2 gene expression, and transcription factors EGR1 and NRF1 could together could synergistically activate PK2 gene expression.

**Figure 5. Promoter sequence most proximal to transcription start site induces highest level of transcription activity.** Next, to find the promoter region most efficient in stimulating transcription of downstream PK2 gene, we sequentially deleted 250 bases from the 5’ 1kb promoter, eventually leaving only the most proximal -250/+1. Promoter assay analyzing the basal activity of the sequential deletions found 5’ truncation that leaves only 250 bases most proximal to the transcription start site remaining had surprisingly increased basal promoter activity to over 150% compared to the basal activity of 1kb promoter activity (Figure 5A). These promoter truncation studies indicate that 250 bases most proximal to the transcription start site is most effective in activating basal level of transcription, and that negative regulatory elements are speculated to bind to -750/-250 region. Further evidence using HIF1α overexpression plasmid DNA (or vector control plasmid) co-transfected with each of the truncated promoter regions also indicated that -250/+1 region was most effective in
stimulating HIF1α overexpression induced promoter activity (Figure 5B). Collectively, these experiments suggest a core promoter region adjacent to the gene transcription start site which could be effectively activated by HIF1α transcription factor.

Figure 6. PK2 may be a major downstream target of EGR1 transcription factor to mediate EGR1-induced effects during neurotoxic insult.

EGR1 have been found to be neuroprotective during ischemia induced neurotoxic stress (Wenfang Lai et al., 2015), and stimulates tyrosine hydroxylase expression in dopaminergic neurons (Akiba, Jo, Akiba, Baker, & Cave, 2009; Fukuchi et al., 2010; Stefano, Al Sarraj, Rössler, Vinson, & Thiel, 2006; Verma, Lim, Han, Nagarajah, & Dawe, 2007). HIF1α has been shown to be neuroprotective in models of PD and also ischemia (Feng et al., 2014; D. W. Lee et al., 2009; Y. C. Lee, Chang, Wu, & Huang, 2018; Lomb et al., 2007). We first wanted to assess the functional effect of EGR1 downregulation during neurotoxic stress. We created a stable EGR1 knockdown N27 dopaminergic cell line, by using CRISPR-cas9. MTS assay showed that EGR1 knockdown N27 cells were more vulnerable to MPP+ compared to wildtype N27 cells (Figure 6A). Similarly, we created a PK2 knockdown dopaminergic cell line in a fashion similar to the creation of EGR1 knockdown N27 cells. MTS assay showed that PK2 knockdown N27 cells were more vulnerable to MPP+ compared to wildtype N27 cells (Figure 6B).

Since EGR1 knockdown could affect PK2 expression during MPP+ treatment, we then showed that inhibition of EGR1 and HIF1α drastically reduces PK2 gene transcription during MPP+ treatment. Since MPP+ induces EGR1 upregulation (Figure 3) to induce PK2 upregulation (Figure 1, Figure 4), we next wanted to test whether EGR1 knockdown could affect PK2 expression during MPP+ treatment. We engineered a stable EGR1 knockdown
dopaminergic cell line by using lentiviral-mediated transduction of CRISPR cas-9 and gRNA in rat dopaminergic cell line (N27), which has high transduction efficiency.

Quantitative PCR of stable EGR1 knockdown treated with control or MPP⁺ saw dampened MPP⁺-induced PK2 response in EGR1 knockdown cells, and showed that that EGR1 knockdown disrupted normal PK2 gene expression during neurotoxic stress. (Figure 6A). With MPP⁺ treatment, scrambled control N27 cells had increased PK2 gene expression of approximately 3.5-fold of control 6h post-treatment, as expected, while PK2 expression in EGR1 knockdown N27 cells was approximately 2-fold of control level at 6h post-treatment (Figure 6A), suggesting that EGR1 knockdown disrupted normal PK2 gene expression during neurotoxic stress. However, the time-course study with EGR1 knockdown cells revealed that MPP⁺-induced PK2 mRNA expression was not reduced in the early stages of neurotoxicity (up to 6h) and was only significantly reduced in later stages of neurotoxicity (6-12h) (Figure 6A). Therefore, it was speculated that another transcription factor was responsible for the early induction of PK2 gene expression.

Then, to simulate a condition that inhibited both EGR1 and HIF1α, EGR1 knockdown N27 was treated with PX-12 (1-methylpropyl 2-imidazolyl disulfide), an anti-cancer compound that reduces HIF1α protein levels (Kim, Coon, Baker, & Powis, 2011; Welsh et al., 2003), to inhibit HIF1α transcriptional activity in neurons. In N27 cells with EGR1 inhibited via knockdown and HIF1α inhibited via PX-12, PK2 gene expression was drastically reduced starting almost immediately at 2h, during which time PX-12 starts to inhibit HIF1α (Figure 6B). Together, these studies suggest a pattern of PK2 gene regulation, with HIF1α inducing immediate PK2 upregulation, and EGR1 participation is required to sustain PK2 upregulation pass the immediate stage into the early stage and onwards. It is likely that HIF1α, EGR1, and
NRF1 transcription factors working in synergy or in combination could therefore achieve high level, sustained PK2 expression.

In summary, EGR1 knockdown disrupted and reduced normal PK2 gene expression during neurotoxic stress, which is found to result in reduced cell viability. Further, the effect of PK2 knockdown on cell viability was similar to the effect of EGR1 knockdown. Considering EGR1 downstream regulation of PK2 gene expression (Figure 4), this suggest that PK2 could be a major downstream target for EGR1.

**Figure 7. PK2, HIF1α, and EGR1 levels are dysregulated during Parkinsonian toxicant-induced neurotoxic stress in mouse model of Parkinsonism.** Manganese (Mn) was used as a Parkinsonian toxicant for in vivo validation of our cell culture findings. Mn-induced parkinsonism has been recognized since 1837 (Couper, 1837; Guilarte, 2010; Racette et al., 2017), and Mn has been used as a mouse model for PD since 1973 (Villalobos et al., 2009). Fundamental cellular pathology such as mitochondrial dysfunction and oxidative stress occurring during Mn-overexposure are strikingly similar to these underlying neurodegenerative processes which also occurs in PD. To test if PK2, HIF1α, or EGR1 are involved in Mn-induced neurotoxicity, we treated Swiss Webster mice with oral gavage of Mn (30mg/kg/day) for 30 days (Figure 8A). At the end of the treatment, mice were sacrificed, and the striatum, substantia nigra (SN) were dissected from the brain.

Western blot analysis of brain tissue found that in the SN, Mn treatment induced PK2 upregulation as well as HIF1α upregulation, while EGR1 levels remained steady (Figure 8B). Surprisingly, in the striatum, PK2 is significantly downregulated, with co-downregulation of both HIF1α and EGR1 (Figure 8C). It is noteworthy that the most significant cell death has been reported in the striatum and the adjacent globus palladus during Mn toxicity
(Latchoumycandane et al., 2005; Olanow, 2004; Peres et al., 2016). Together, these studies indicate that PK2 expression is largely co-regulated with the expression of HIF1α and EGR1 in vivo during neurotoxic stress.

In summary, EGR1, HIF1α, NRF1, and PK2 upregulation are induced in response to neurotoxicant MPP+ or Mn treatment in cell culture studies. Promoter studies show that EGR1, HIF1α, and NRF1 could induce PK2 promoter activity, gene transcription, and protein upregulation. EGR1 knockdown and HIF1α inhibition studies indicates a dampened PK2 upregulation in response to MPP+. Together, these studies suggest that neurotoxic stress could induce the upregulation of EGR1 and HIF1α which then stimulate the downstream PK2 as compensatory protective response, by transcriptionally activating PK2 gene expression. Furthermore, initiation of high level, sustained PK2 expression likely involves several transcriptional factors working in synergy or in combination.

Discussion

We had previously shown that PK2 could mitigate the production of ROS, bolster mitochondrial biogenesis, and counter pro-apoptotic signals in dopaminergic neurons. Despite these recent advances in understanding of PK2’s functions, its role and its transcriptional regulation during neurotoxic stress remain incompletely understood. In this study, we analyzed the PK2 proximal promoter for clues of its transcriptional activation. Using the in silico analysis as a starting point, we found putative transcription factors EGR1, NRF1, HIF1α, and E2F could respond to neurotoxic stress in dopaminergic cell cultures, and further validated their effects on PK2 promoter activity. Using CRISPR cas9-based knockdown and chemical inhibition of HIF1α, we found that EGR1 and HIF1α are major regulators of PK2 gene expression during MPP+-induced PK2 upregulation. Lastly, we found that PK2 expression
generally co-regulated with EGR1 and HIF1α in Mn overexposure animal models of neurotoxic stress.

Since the discovery of 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) as a potent dopaminergic system-specific neurotoxicant in human patients around 1987, it has been used to create PD models in animals (Langston, 1987). Its functional metabolite, MPP⁺, has since been used in cell culture to for dopaminergic neurotoxicant as a potent mitochondrial complex I inhibitor. The accumulation of MPP⁺ causes the collapse of mitochondrial potential, activation of caspase-3, and the ensuing apoptosis.

Successes against MPTP-induced dopaminergic neuronal cell loss and striatal innervation have been found via treatment with FG-4592 and 3,4-dihydroxybenzoate (DHB) (D. W. Lee et al., 2009; X. Li et al., 2018), both prolyl hydroxylase inhibitors and hence HIF1α stabilizers. Treatment with DHB was shown to rescue mitochondrial dysfunction, a major mechanism of DHB-mediated protective effects. Treatment with FG-4592 produced similar effects. Our previous finding indicated that PK2 could induce proliferator-activated receptor-γ coactivator 1α (PGC-1α) in the activation of mitochondrial biogenesis and respiration, therefore it could be speculated that HIF1α activation of PK2 could help in mediating PGC-1α’s protective effects. Further studies need to be conducted to confirm the mechanism of a putative HIF1α-PK2-PGC-1α axis. Additionally, we found that HIF2 overexpression could induce PK2 promoter activity. Molecular suppression of HIF-2α increases the basal sensitivity to oxidative death (Nanduri et al., 2009). HIF2 governs prolonged hypoxic gene activation (Holmquist-Mengelbier et al., 2006) and promotes iron absorption in mice (Mastrogiannaki et al., 2009). Interestingly, MTF1, a transcription factor with a putative binding site on the PK2 promoter, is involved in metal homeostasis in response to heavy metals such as cadmium, zinc,
copper, and silver. These transition metals induce MTF-1 translocation into the nucleus to bind to the Ferroportin1 (FPN1) promoter (Troade, Ward, Lo, Kaplan, & De Domenico, 2010). The dysregulation of FPN1 has recently been implicated in the pathogenesis of PD (Xu, Kanthasamy, Jin, & Reddy, 2016).

Several lines of evidence suggest that EGR1 is important for neuronal plasticity, dopaminergic neuronal maintenance and tyrosine hydroxylase expression (Kress & Wullimann, 2012; Papanikolaou & Sabban, 2000). Its upregulation is attributed to a number of neuroprotective regimens ranging from the anti-ROS actions of resveratrol (Han, Zheng, Bastianetto, Chabot, & Quirion, 2004) to anti-inflammatory, anti-oxidative, and anti-apoptotic properties of salidroside (W. Lai et al., 2015), and its downregulation has been associated with decreased cognition in dementia (Gersten et al., 2009). In addition, EGR1, EGR2, EGR3, and EGR4 are co-upregulated with brain-derived neurotrophic factor (BDNF) after treatment with pridopidine in Huntington disease mouse models (Kusko et al., 2018). Furthermore, erythropoietin (EPO), a neuroprotective cytokine in models of ischemic injury, induced EGR1, EGR2, EGR4 expression and directly activated EGR2 (Mengozzi et al., 2012). Importantly, MAPK/ERK signaling pathway could activate EGR1 to stimulate neurite outgrowth in a traumatic brain injury model (Chasseigneaux et al., 2011; Plummer, Van den Heuvel, Thornton, Corrigan, & Cappai, 2016). Lastly, EGR1 activates high levels of glial-cell derived neurotrophic factor (GDNF), a neurotrophic factor necessary for maintenance of dopaminergic neurons, by directly binding to the Gdnf promoter (Shin et al., 2009).

The presence of putative binding site for neuron-restrictive silencer factor could suggest that PK2 might be involved in processes of axon sprouting in neurons. NRSF represses expression of neuronal genes in nonneuronal cells and in neuronal progenitor cells (NPC)
(Huang, Myers, & Dingledine, 1999; Jones & Meech, 1999). NRSF binds to target genes and recruit histone deacetylases, making DNA more basic and tighter association of histone to DNA thereby less accessibility to transcription factors (Chen, Paquette, & Anderson, 1998; Naruse, Aoki, Kojima, & Mori, 1999). Through this mechanism, NRSF also negatively regulates the activity of neuropilin-1, which is a positive regulator of axon branching promoted by the actions of VEGF and EGF (Kurschat, Bielenberg, Rossignol-Tallandier, Stahl, & Klagsbrun, 2006). These lines of evidence suggest that this balancing control aims to prevent the expression of PK2 and other co-regulated neurogenesis-associated genes such as neuropilin-1 until the precise moment that they are needed. In the same vein, vertebrate homologues of enhancer of split complex (HES) is another bHLH TF that was predicted to bind to the PK2 promoter. HES1 is critically important for neurogenesis (Dearden, 2015); decreasing expression of HES promote the expression of proneural genes, while reducing the pool of neural stem cell. Thus, HES1 works to provide a delicate balance between neurogenesis of new neurons and maintenance of neural stem cell pool, by repressing proneural identity. It has been known that PK2 is indispensable for olfactory bulb neurogenesis owing to its chemotactic properties. However, the expression of PK2 is differentially upregulated as neuronal progenitor cells differentiate into functional neurons, and the upstream events which results in the cell’s decision to precisely upregulate PK2 at the most opportune moment remains unclear. Factors such as NRSF and HES involved in suppressing neuronal genes could provide such fine control of PK2 expression.

Deletion of the -750/-250 region of the promoter increases promoter activity, indicating possible negative regulatory elements in this region. Given PK2’s compensatory, protective role in neurodegeneration, this represents a target for PK2 transcriptional regulation and
therapeutic intervention. Understanding the upstream transcription factors induced by neurotoxic stress that activate protective, compensatory PK2 upregulation is crucial in devising a potential strategy for slowing and reverse the progression of neurodegeneration such as PD.

**Materials and Methods**

**Promoter Cloning and Promoter Assay.** Three kilobases of PK2 promoter sequence contained in a proprietary plasmid was obtained from Switchgear promoter company and verified through sequencing. One kilobase of the promoter sequence upstream of the predicted transcription start site was then subcloned into Promega’s pNL1.1 luciferase vector. Luciferase promoter assay: MN9D cells were grown in 12 well plates overnight and co-transfected with PK2 promoter plasmid (pNL1.1-PK2) and selected transcription factors. Cells were harvested and prepared according to Promega Nanoluc Luciferase Assay kit protocol and the promoter assay was performed using manufacture’s protocols.

**Animal handling.** All animal procedures were approved by Iowa State University’s Institutional Animal Care and Use Committee (IACUC). All mice were housed under a 12-h light cycle in a climate-controlled mouse facility (22±1 °C) with food and water available ad libitum. Male C57B/6 mice were pre-screened during behavioral assessments for normal baseline performance before being randomly assigned to experimental groups. Animals were not wounded or otherwise exhibited conditions that could affect behavior, and are taken off the study if any conditions have manifested which could affect behavioral measurements. Investigators involved with data collection and analysis were not blinded to group allocation.

**Behavior monitoring.** All groups were monitored for behavior using the automated VersaMax system software connected to motion-detection hardware (VersaMax monitor, model RXYZCM-16, and analyzer, model VMAUSB, AccuScan, Columbus, OH). Each
animal was put in one chamber fitted with motion-detection hardware, and allowed to acclimate for 2 minutes, after which its spontaneous locomotor activities (horizontal activity, vertical activity, and speed) were recorded for 10 minutes.

**Western blot.** Dissected brain regions were collected in Eppendorf tubes and flash frozen. To isolate total protein, RIPA buffer with sodium orthovanadate, Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher 78440) was added to each tube, and homogenized using a tissue homogenizer. Dissolved total lysate was centrifuged at 12,000g for 60 minutes, to remove cellular debris. Normalized protein samples were loaded into each well and were separated by sodium dodecyl sulfate (SDS) gel electrophoresis (100V, 90 minutes), using AnykD Mini-PROTEAN resolving gel (Bio-Rad, #4569035). Proteins on SDS gels were then transferred to a nitrocellulose membrane (26V, overnight) and blocked for 1h using fluorescent western blocking buffer (Rockland Immunochemicals). Primary antibodies were diluted in blocking buffer with 0.05% Tween 20, were then added to the membranes and incubated overnight at 4 °C. Next day, primary antibodies were taken off, and blots were washed (7 times, 5 minute each) in wash buffer comprising PBS containing 0.05% Tween 20 (PBST). Secondary antibody (infrared dye-tagged) was added for 1 h. Blots were further washed in PBST for 3 more times, and once in PBS. β-Actin was used as a loading control. Membranes were scanned using the Odyssey IR imaging system (LI-COR) and digital images were captured via LI-COR Odyssey imager. Densitometric analysis was done using ImageJ software.

**Immunohistochemistry.** Cells were fixed in 4% paraformaldehyde blocked with BSA and Triton-X to permeabilize the cell membranes. Primary antibodies were used as follows: rabbit anti-TH (1:1600 dilution), rabbit anti-PK2 (1:500 dilution), goat anti-GDNF (1:500
dilution), goat anti-IBA1 (1:1000), rabbit anti-GFAP (1:1000 dilution). Alexa Fluor Fluorescent Secondary Antibodies from ThermoFisher were used against primary antibodies for fluorescent images. For DAB staining, HRP-conjugated secondary antibodies were used against primary antibodies, and VECTASTAIN Elite ABC HRP Kit (VectorLabs) was used for conjugation of HRP to secondary antibodies. DAB was used for color development.

**SYBR Green qRT-PCR.** To obtain total RNA, tissue lysis buffer from Absolutely RNA Miniprep kit (Agilent Technologies) was added to dissected brain regions with addition of beta-mercaptoethanol as reducing agent to preserve RNA. A tissue homogenizer was used to dissolve tissue into the lysis buffer, and lysates were processed according to kit manufacturer’s instructions. Isolated total RNA from each sample was quantified using Nanodrop instrument to determine RNA concentration and purity. First strand cDNA synthesis was performed using Affinity Script qPCR cDNA synthesis system (Agilent Technologies) with 1µg of total RNA in the reaction mixture. Real-time PCR was performed with the RT^2 SYBR Green master mix (Qiagen) using diluted cDNA, and qPCR mouse primer sets were purchased from Qiagen (Quantitect primer mix). The 18S rRNA gene (mouse) was used as normalization of each sample as the housekeeping gene. The amount of each template was optimized empirically to maximize efficiency without inhibiting the PCR reaction. After the last cycle of the qPCR run, dissociation curves were run to ensure a single amplicon peak was obtained, indicating primer specificity. The results are reported as fold change in gene expression with the ΔΔCt method, using the threshold cycle (Ct) value for the housekeeping gene and for the respective gene of interest in each sample. Control animals serve as the baseline for fold change.
**Lentivirus production.** PK2 lentivirus expression vector was obtained from Origene, and the MISSION Lentiviral Packaging Mix was obtained from Sigma (SHP001) and used according to manufacturer’s protocols. Briefly, the PK2 lentivirus expression vector was mixed with the Packaging Mix, and co-transfected into 293FT cells to package the virus. After 24h post-transfection, the supernatant, which contain the virus, was collected. The second harvesting of virus was done 48h post-transfection. The Lenti-X™ p24 Rapid Titer Kit from Clontech (632200) was used to titer the lentivirus.

**Acknowledgements**

HIF1α PAS mutant was a kind gift from Dr. Cathy Miller.

**References**


Figures

**A.** Neurotoxicant MPP⁺ upregulates PK2 and induces PK2 promoter activity in dopaminergic neurons. Parkinsonian toxicants MPP⁺, Mn, as well as HIF1α stabilizer DHB, could induce PK2 mRNA expression as found by quantitative PCR. MN9D cultured mouse dopaminergic neurons were treated with MPP⁺, Mn, or DHB dissolved in ddH2O. After 4h of treatments, cells from each flask were harvested and processed for RNA isolation. **B.** Quantitative PCR assay also showed that a MPP⁺ treatment at 300µM upregulates PK2 gene
expression during early neurotoxic stress, peaking at 3 hours post treatment. MN9D cultured mouse dopaminergic neurons were treated with MPP\(^+\) dissolved in ddH\(_2\)O. After the respective time points (0h, 1h, 3h, and 12h) cells from each flask was harvested and processed for RNA isolation. C. Western blot assay (top) and densitometric analysis (bottom) showed early increases in PK2 protein level at 3h, 6h, and 12h-post treatment. MN9D cells were treated and harvested similarly, and processed for western blot. Beta-actin was used as internal loading control. D. MPP\(^+\) increased PK2 promoter activity in MN9D dopaminergic cell host transiently transfected with the PK2 promoter construct, increasing luciferase activity at 3h and 6h-post treatment by 2-fold. MN9D cells were transfected the previous day with PK2 promoter construct, and 24h later, treated with MPP\(^+\) for 0h, 1h, 3h, 6h. Cells were harvested at designated timepoints and processed for promoter reporter assay.
Figure 2. In silico analyses reveal conserved, methylated sequences 1kb upstream of proximal PK2 promoter, and a wide range of transcription factors associated with metabolic processes, cellular processes, cellular biogenesis, and transcription factors regulating response to stimuli. **A.** Using ECR Browser to find conserved sequences between human PK2 promoter sequences and other species, 1kb upstream of the transcription start site has been found to be
conserved across rodents and canines. **B.** Analysis of promoter CpG islands using EMBOSS CpGplot found increased CpG around -400 to +1 relative to transcription start site. **C.** MethPrimer analysis found two CpG islands near -400 to +1 of the transcription start site. **D.** Upstream 1kb promoter sequences was uploaded into the MatInspector software from Genomatix. Transcription factors are summarized in Table 1 and Table 2. **E, F.** Pie chart and histogram of broad categories of genes found in Table 1, as classified by the PANTHER Classification System for Gene Ontology. **G, H.** Pie chart and histogram of broad categories of genes found in Table 2, a shorter list of genes expressed in the CNS, as classified by the PANTHER Classification System for Gene Ontology.
Figure 3. PK2 may be a target gene for Early growth response family of transcription factors (EGRs), Nuclear respiratory factor 1 (NRF1), Hypoxia-inducible factor 1 (HIF1α), and E2F-myc activator/cell cycle regulator. A. Putative transcription factor core binding sites on 1kb PK2 promoter. B. Western blot analysis showed that MPP⁺, manganese, or hydrogen peroxide drastically increased EGR1 protein level within 6h post-treatment. C. PK2 mRNA was rapidly induced by MPP⁺ within 30 minutes and is sustained at 6h. These data suggest that EGR1 is responsive to neurotoxic stress.
Figure 4. Overexpression of EGRs, NRF1, HIF1α, or E2F1 in MN9D dopaminergic neurons induces PK2 promoter activity. A. Promoter reporter assay revealed that co-transfection of 1kb PK2 proximal promoter nano-luciferase reporter construct (3ug) with plasmid vector encoding wildtype EGR1, EGR2, EGR3, could induce PK2 promoter activity. Members of the EGR family could stimulate PK2 promoter activity, with approximately 400% increase for EGR1 overexpression, approximately 150% increase for EGR2 overexpression, and over 1000% for EGR3 overexpression. B. NRF1 also stimulated promoter activity by approximately 550%. C. HIF1α overexpression moderately but significantly induced PK2 promoter activity. DHB treatment and DHB treatment in combination with HIF1α overexpression produced more than 200% increase in promoter activity. Additionally, mutant
HIF1α with defective PAS domain did not stimulate the PK2 promoter activity. D. Overexpression of HIF2α could stimulate approximately 300% increase in PK2 promoter activity. E. HIF2α Overexpression of E2F drastically stimulated PK2 promoter activity, with nearly 3500% increase. F-I. Analysis using qPCR showed that EGR1 overexpression induced PK2 gene expression approximately 4.5-fold of control, while NRF1 overexpression induced > 3-fold of control, HIF1α overexpression induced > 4-fold of control, and E2F overexpression induced > 5-fold of control.
Figure 5. Promoter sequence most proximal to transcription start site induces highest level of transcription activity. 

A. Promoter assay analyzing the basal activity of the sequential deletions found that 5’ truncation of 250 bases reduced promoter activity to approximately 50% of 1kb promoter, while a further 5’ truncation of 250 bases marginally restored the basal promoter activity. The -250/+1 promoter sequences most proximal to the transcription start site had surprisingly increased basal promoter activity to over 150% compared to the basal activity of 1kb promoter activity. 

B. HIF1α overexpression plasmid DNA (or vector control
plasmid) co-transfected with each of the truncated promoter regions also indicated that HIF1α stimulated 200% promoter activity when co-transfected with -750/+1 truncated promoter, and strong activation at close to 250% was seen comparatively when co-transfected with -500/+1 truncated promoter, and nearly 300% as compared to vector control plasmid.
Figure 6. PK2 may be a major downstream target of EGR1 transcription factor to mediate EGR1-induced effects during neurotoxic insult. A. MTS cell viability assay showed that EGR1 knockdown exacerbates MPP⁺-induced cell death. While treatment with 300µM MPP⁺ resulted in approximately 60% viability after 24h in wildtype N27 cells, only approximately 40% of N27 cells were viable after EGR1 knockdown. B. MTS cell viability assay showed that PK2 knockdown also exacerbates MPP⁺-induced cell death, with cell survival similar to EGR1 knockdown. C. Inhibition of EGR1 drastically reduces PK2 gene transcription. PK2 gene expression in scrambled control or EGR1 knockdown N27 cells were...
treated with control or MPP+. EGR1 knockdown in rat dopaminergic cell line, N27 cells, was made by using lentiviral-mediated transduction of CRISPR cas-9 and gRNA. The qPCR analysis showed that basal level of PK2 gene expression reduced approximately 30% in EGR1 knockdown N27 cells. With MPP+ treatment, scrambled control N27 cells had increased PK2 gene expression of approximately 3.5-fold of control 6h post-treatment, as expected, while PK2 expression in EGR1 knockdown N27 cells was approximately 2-fold of control level at 6h post-treatment. D. Inhibition of EGR1 and HIF1α together drastically reduces PK2 gene transcription. EGR1 knockdown N27 was treated with PX-12 to inhibit HIF1α transcriptional activity in neurons. In N27 cells with EGR1 inhibited via knockdown and HIF1α inhibited via PX-12, PK2 gene expression was drastically reduced starting almost immediately at 2h.
Figure 7. PK2, HIF1α, and EGR1 levels are dysregulated during Parkinsonian toxicant-induced neurotoxic stress in mouse model of Parkinsonism. 

**A.** Treatment paradigm of Mn, which was used as a Parkinsonian toxicant for in vivo validation of our cell culture findings. Swiss Webster mice was treated with oral gavage of Mn (30mg/kg/day) for 30 days. At the end of the treatment, mice were sacrificed, and the striatum, substantia nigra (SN) were dissected from the brain. 

**B.** Western blot analysis of SN (top panel) and striatum (bottom panel), in mice treated with Mn. In SN, PK2, and HIF1α are upregulated, while EGR1 levels remained steady. In the striatum, PK2 is significantly downregulated, with co-downregulation of both HIF1α and EGR1.
CHAPTER 3. PROKINETICIN 2 PROTECTS AGAINST MANGANESE-INDUCED NEUROTOXICITY BY INDUCING MITOCHONDRIAL BIOGENESIS IN DOPAMINERGIC CELLS

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Abstract

Over exposure to manganese (Mn) causes manganism, a neurological disorder with symptoms similar to Parkinson's disease. However, the cellular mechanism by which Mn impairs the dopaminergic neurotransmitter system remains unclear. While much focus has been placed on cell death processes underlying the neurotoxic effect of Mn, early neuronal response to Mn exposure deserves more attention. Here we found that the dopaminergic neurotoxicant Mn rapidly induce PK2 during the early stages of Mn neurotoxic stress in an N27 dopaminergic cell model. To better understand the functional role of PK2 upregulation, we created stable PK2-expressing dopaminergic cells by delivering PK2 myc-tagged cDNA into mouse dopaminergic MN9D cells. Interestingly, PK2-overexpressing cells exposed to manganese showed significant protection against neurotoxicity relative to vector control cells, suggesting a neuroprotective role for PK2 in dopaminergic neurons. The protective effect was both dose- and time-dependent. Furthermore, the PK2 receptor blocker PC7 attenuated the PK2-induced neuroprotective effects in PK2-overexpressing cells. Overexpressing PK2
protected against manganese-induced apoptosis as measured by Annexin 5 and caspase-3 activation. We also found that mitochondrial integrity was well maintained in PK2-overexpressing cells relative to vector cells following exposure to Mn. Preliminary results also showed that key proteins involved in mitochondrial functions, including BCL2, PGC1-α and TFAM levels, were preserved in PK2-overexpressing cells during neurotoxic stress. Collectively, our results suggest that neurotoxic insults upregulate PK2 in dopaminergic neurons to protect against the early stages of neurotoxicity. Finally, observed the effect of Mn treatment on PK2 expression using a GENSAT PK2 GFP transgenic mouse model. During early exposure to Mn (30 mg/kg for 10 days, oral), PK2 levels were significantly upregulated in the SN while slightly decreasing in striatum. Prolonged exposure to Mn (30 mg/kg for 30 days) significantly upregulated PK2 levels in the brain, especially in the substantia nigra. Interestingly, in the striatum, where Mn-induced cell death mainly occurs, decreased PK2 levels were noted. Significant amount of methylation of the PK2 promoter region is also observed in the striatum. Combined with cell culture studies, the differential regulation of PK2 in the striatum and substantia nigra might suggest a possible neuroprotective role of PK2 in the SN during early exposure to Mn. Taken together, these results suggest that Mn upregulates PK2 levels to counter early neurotoxic stress in vitro and in vivo.

Introduction

Manganese (Mn) is an essential trace element in all known living organisms. It is required for cellular maintenance and participates in such fundamental cellular processes as metabolism of fats and carbohydrates, regulation of blood sugar, and calcium absorption (Erikson, Syversen, Aschner, & Aschner, 2005). It is also a cofactor for glutamine synthetase, arginase, pyruvate decarboxylase, serine/threonine phosphatase, and superoxide dismutase 2
Mn exists in various chemical forms including several oxidation states (Mn$^{2+}$, Mn$^{3+}$, Mn$^{4+}$) (Rask, Miner, & Buseck, 1987; Reaney & Smith, 2005), a versatile chemical property which enabled its industrial usage in making glass and ceramics, adhesives, welding, paint, gasoline anti-knock additives” and medical device batteries (Loranger & Zayed, 1995; Nduka, Onyenezi Amuka, Onwuka, Udowelle, & Orisakwe, 2016; Sriram et al., 2014).

Wide range of industrial uses consequently increases risks of overexposure to Mn for workers such as welders and miners in occupational settings. Routes of overexposure are mainly through dermal absorption and inhalation. Despite its essential role in multiple metabolic functions, Mn can accumulate in the brain, a major organ of Mn toxicity during overexposure. Excessive Mn efficiently crosses the blood brain barrier and blood-cerebrospinal fluid barrier, to preferentially concentrate in globus pallidus and striatum in monkeys (Dastur DK, Manghani DK, 1971; Fujii, 1975) and humans (Aschner, 2006; Aschner, Guilarte, Schneider, & Zheng, 2007; Sarkar et al., 2018; Wooten, Aweda, Lewis, Gross, & Lapi, 2017; Yokel, 2009). The disruption of the nigrostriatal pathway of the basal ganglia system in prolonged Mn overexposure causes a severe neurological disorder similar to Parkinson’s disease named manganism (Cawte, Hams, & Kilburn, 1987). manganism is characterized clinically by extrapyramidal symptoms resembling Parkinson’s disease, with depression, postural instability, bradykinesia, micrographia, and a characteristic cock-walk caused by dystonia of the legs (Cersosimo & Koller, 2006; Olanow, 2004; Perl & Olanow, 2007). Tremor is less commonly seen compared to Parkinson’s disease, and are postural or kinetic in nature, rather than resting (Casamajor, 1913; Mena, Marin, Fuenzalida, & Cotzias, 1967). Furthermore, Mn overexposure are more detrimental to younger individuals, who
generally accumulate greater Mn levels in the CNS (Campbell, 2005; Grandjean & Landrigan, 2006; Rodier, 1995; Zheng, Aschner, & Ghersi-Egea, 2003). They are more vulnerable to its toxicity during brain development, and may be predisposed to later-life neurological disorders by Mn overexposure (Erikson, Thompson, Aschner, & Aschner, 2007; Hafeman, Factor-Litvak, Cheng, van Geen, & Ahsan, 2007; Moreno, Streifel, Sullivan, Legare, & Tjalkens, 2009). Neuropathologically, it is characterized by lesions found in globus pallidus and striatum downstream in the nigrostriatal pathway (Olanow, 2004; Perl & Olanow, 2007), causing nigrostriatal disruption and extrapyramidal symptoms (ATSDR Toxicological Profile for Mn).

The effects of Mn on the dopaminergic system remain controversial and the cellular mechanism by which Mn impairs the dopaminergic neurotransmitter system remains unclear in adults and children (El-hady & Galal, 2018; Gandhi, Sivanesan, & Kannan, 2017; Lao et al., 2017). Some studies suggested increase in dopamine with acute exposures of Mn, while chronic exposures to Mn in experiments using nonhuman primates as well as PET scans of welders suggested that Mn could inhibit the normal function of dopaminergic neurons by marked inhibition (> 50% on average) dopamine release in the striatum. Such prolonged exposure then causes cell death in the globus pallidus of the striatum (Perl & Olanow, 2007).

Mn can be transported into the cell via the actions of the divalent meta transporter DMT1, the transferrin receptor (Tfr), and SLC39 zinc transporters, as well as ATP13A2, among which DMT1 and Tfr are the most well-studied (Aschner & Aschner, 1990; Eide, 2004; Heilig et al., 2006; Kim et al., 2015). Within the cell, Mn causes impairment in iron homeostasis, excitotoxicity, oxidative stress, and mitochondrial dysfunction (Afeseh Ngwa et al., 2011; Harischandra, Jin, Anantharam, Kanthasamy, & Kanthasamy, 2015; Sarkar et al., 2018). A major cellular storage of Mn is the mitochondria, where Mn preferentially
accumulates via the Ca\(^{2+}\) uniporter. At excessive levels, Mn causes dissipation of the mitochondrial membrane potential (Allshire, Bernardi, & Saris, 1985; Gavin, Gunter, & Gunter, 1999; Rao & Norenberg, 2004). In detailed electron microscopy micrographs, neurons in mice nasally administered Mn for one month showed swollen mitochondria, and disorganization of the inner membrane system (Villalobos et al., 2009). Mn exposure has been used as a mouse model for PD since 1973 (Villalobos et al., 2009), with mitochondrial dysfunction and oxidative stress occurring in manganism being strikingly similar to the underlying neurodegenerative processes occurring in PD. This suggests that toxicity involving mitochondria could be common in both manganism and PD.

Furthermore, recent studies had found Mn overexposure could induce aberrant epigenetic changes such as changes in histone acetylation (Guo et al., 2018), DNA hypermethylation or hypomethylation during development for genes regulating neurogenesis (Wang et al., 2013) and during neurodegeneration for genes regulating cell survival (Chahrour et al., 2008; A. Kanthasamy et al., 2012; Tarale et al., 2016, 2017; Wang et al., 2013; Yang Qiao, Hua Shao, Jack Ng, 2015). Therefore, downregulation of key protective genes caused by aberrant methylation could be a major culprit in Mn-induced neurotoxicity.

Prokineticin-2 (PK2) is a secreted neuropeptide that binds to two cognate receptors prokineticin receptor 1 (PKR1) and prokineticin receptor 2 (PKR2). The PK2 signaling cascade through PKR1 or PKR2 regulates diverse physiological processes including hematopoiesis, reproductive functions, pain perception and angiogenesis in the peripheries. In the brain, PK2 is a chemoattractant during olfactory bulb neurogenesis, and is also an output molecule of the suprachiasmatic nucleus in the hypothalamus, oscillating in levels of expression to transmit circadian rhythm into nearby structures (Cheng et al., 2002; Hu et al., 2006; Li et al., 2006;
Lin et al., 2002; Ng et al., 2005; Qun Yong Zhou & Cheng, 2005). Recently we have reported that PK2 is upregulated in human PD patient post-mortem brains. We showed that in MPTP-induced and MitoPark genetic mice models of PD, PK2 is upregulated as a compensatory response to protects against dopaminergic degeneration (Gordon et al., 2016). Importantly, we previously found that PK2 protects against oxidative stress, mitochondrial dysfunction, and promotes mitochondrial biogenesis, by reducing mitochondrial fragmentation, increasing mitochondrial length and number, and bolstering expression of TFAM, PGC1α.

Since mitochondrial dysfunction is central in both PD and manganism, elucidating the role of PK2 in mitochondria during Mn toxicity could shed light on mitochondrial dysfunction in both pathological conditions. We hypothesized that PK2 might be involved in compensatory responses in early Mn-induced neurotoxicity. In the current study, we found that PK2 is upregulated during Mn-induced neurotoxicity, and showed that PK2 is a compensatory protective response against Mn-induced neurotoxicity in cell cultures. PK2 overexpression conferred protection against oxidative stress, enhanced mitochondrial biogenesis, while blocking PK2 signaling nullified protective effects. Importantly, we found that PK2 is upregulated in the substantia nigra of mice during subacute and subchronic exposures of Mn, but is downregulated in the striatum, possibly due to PK2 promoter hypermethylation in the striatum induced by Mn toxicity.

Although progress in understanding of mechanisms of toxicity for Mn overexposure has been made since its initial observation in James Couper in 1837, currently there is no cure against manganism (Chen, Chakraborty, Peres, Bowman, & Aschner, 2015; Dobson, Erikson, & Aschner, 2004; Nielsen, Larsen, Ladefoged, & Lam, 2017; Tarale et al., 2016; Tjalkens, Popichak, & Kirkley, 2017). The situation is made dire due to a lack of response to L-DOPA
for motor symptoms present in manganism patients (Bowler, Koller, & Schulz, 2006; Koller, Lyons, & Truly, 2004). Current developments of potential therapies in preclinical testing involve stem cell transplantation strategies (de Moura, Afadlal, & Hazell, 2018). Given neuroprotective properties of PK2, differential regulation of PK2 leading to downregulation of PK2 in the striatum could represent a mechanism of Mn toxicity as well as a point of intervention as therapies for manganism patients.

Results

**Figure 1. Neurotoxicant Mn treatment induces PK2 upregulation in N27 dopaminergic cells.** From a neuroinflammation PCR array, we initially had found that the expression of PK2 in dopaminergic cells treated with TNFα 7-fold was increased 7-fold. Thereafter, we had discovering its role in mediating cell survival in Parkinsonian toxicant MPP⁺-induced dopaminergic neuron degeneration. We then hypothesized that PK2 could also mediate neuronal survival in Mn-induced neurotoxicity. We first treated cultured N27 rat dopaminergic neurons with a biologically relevant dose of Mn, 300µM in the form of MnCl₂, and the classic Parkinsonian toxicant MPP⁺, as well as a HIF1α stabilizer, 3,4-dihydroxybenzoate (DHB). Mn treatment induced PK2 mRNA expression 4h post-treatment of Mn as assessed by qPCR (Figure 1A). Mn was able to induce sustained increase in protein levels of PK2 at 6h to 12h post treatment (Figure 1B). This suggests that PK2 signaling is implicated in Mn-induced cellular responses.

**Figure 2. Stable PK2 overexpression in dopaminergic cell line protects against Mn-induced cytotoxic cell death.** We engineered MN9D mouse dopaminergic cell line to overexpress PK2 by stable transfection of plasmid vector carrying human PK2 cDNA and a small 1.2 kD myc-tag, to confirm the role of PK2 during Mn-induced neurodegeneration.
Representative western blot and densitometric analysis of 3 western blot runs for human PK2 indicates high expression in MN9D dopaminergic cells after stable transfection (Figure 2A, 2B). Immunostaining of PK2-myc using antibody against the myc tag found increased PK2-myc in the cytosol (Figure 2C). PK2, as a signaling neuropeptide, can be secreted outside of the cell (Bullock, Li, & Zhou, 2004; Q. Y. Zhou & Meidan, 2008). Coincidentally, immunostaining for the myc tag also found significant amount of PK2-myc in the Golgi apparatus, possibly undergoing posttranslational modifications for secretion outside of the cell. Since high constitutive overexpression could lead to receptor downregulation, we assessed level of PKR2, a receptor for PK2 on dopaminergic neurons, in vector and PK2 overexpressing cells. Interestingly, western blot immunostaining of PKR2 found that it is not affected in its protein levels by PK2 overexpression, indicating that constitutive overexpression of PK2 does not lead to unwanted downregulation of the receptor (Figure 2D).

After establishing the stable PK2 overexpression dopaminergic cell line, in our next set of studies, we assessed the protective effects of PK2 overexpression during Mn-induced neurodegeneration. Vector and PK2 overexpression cells were treated with increasing doses of Mn ranging from 0µM to 500µM which were added to growth media with reduced serum (2% FBS) for 24 hours. MTS assay analysis showed that while PK2 overexpression caused little change in cell viability in control conditions without Mn treatment, PK2 overexpression significantly increased cell metabolic activity associated with cell viability in almost all doses of Mn (Figure 2E). Cell viability in PK2 overexpression cells was close to 15% higher as compared to vector cells, when treated with 300µM Mn for 24 hours (Figure 2E). However, blocking with PC-7, a GPCR blocker, abolished any protective effects afforded by PK2 overexpression (Figure 3F). Flowcytometry analysis showed that Mn 300µM treatments, in
vector cells induced ANNEXIN-5 in approximately 55% of vector cells, in comparison to 35% of PK2-overexpressing cells, indicating less PK2 overexpression cells undergoing apoptosis when treated with Mn (Figure 2G). This set of experiments indicates that constitutive PK2 expression could protect neurons during Mn-induced stress, and the protective effects are mediated through secreted PK2.

Figure 3. Stable PK2 overexpression in dopaminergic MN9D cells protects against Mn by reducing caspase activation and increasing BCL-2 levels. Our next set of experiments attempted to elucidate the mechanism of PK2 overexpression-mediated protection. Caspase-3 activation is a major pathway of apoptosis that could lead to PKCδ proteolytic cleavage and apoptotic cell death of dopaminergic neurons exposed to Mn (Anantharam, Kitazawa, Wagner, Kaul, & Kanthasamy, 2002; Latchoumycandane et al., 2005). By using a fluorescent, substrate-based assay, caspase-3 activity was found to drastically increase in vector cells 16h post-Mn treatment, while PK2-overexpression reduced caspase-3 activity to close to caspase-3 activity levels in control cells (Figure 3A). Since upregulation of Bcl-2 could promote anti-apoptotic effects in dopaminergic neurons during toxic insult (Gordon, Anantharam, Kanthasamy, & Kanthasamy, 2012; A. G. Kanthasamy et al., 2003; Kitazawa et al., 2005a; Kitazawa, Anantharam, Kanthasamy, & Kanthasamy, 2004), we sought to determine the expression of Bcl-2 in vector or PK2 overexpression cells treated with or without Mn. We found PK2 overexpression significantly increased gene expression of Bcl-2 in qPCR assay (Figure 3B). While Mn treatment significantly reduced Bcl-2 gene expression, PK2 overexpression preserved Bcl-2 gene expression in cells treated with Mn for 8h (Figure 3B). Western blot assay (top) and densitometric quantification (bottom) had found
increased Bcl-2 protein levels in PK2 overexpression cells and preserved Bcl-2 protein levels during Mn treatment, while decreasing Bax (Figure 3C-3E).

**Figure 4. PK2 overexpression protects MN9D cells against Mn-mediated mitochondrial dysfunction, while knockdown of PK2 exacerbates Mn-induced mitochondrial dysfunction.** Mitochondria’s role in the etiology of neurodegenerative diseases, as well as a target of intervention, have become increasingly recognized (Dranka et al., 2014; Jin et al., 2014). We had previously found that PK2 could bolster mitochondrial biogenesis against MPP⁺-induced mitochondrial dysfunction, here we found that while Mn treatment for 24h drastically decreases mitochondrial content, PK2 overexpression could restore Mn-induced decreases in mitochondrial content, as stained by MitoTracker dye (Figure 4A). Quantitative-PCR analysis of mitochondrial DNA content (mDNA) showed a significant decrease in mDNA in cells treated with Mn for 24h, while PK2 overexpression significantly increased mDNA in cells treated with Mn (Figure 4B). Western blot analysis showed that after treatment with Mn, PK2 overexpression also preserves mitochondrial levels of PGC1alpha and TFAM, transcription factors which are important regulators of mitochondrial-transcribed genes. (Figure 4C). To assess the effect of PK2 downregulation on mitochondrial function, we generated a N27 rat dopaminergic cell line defective in PK2 expression using a CRISPR-Cas9 lentivirus system. The CRISPR-Cas9 system is driven by the activity of the Cas9 protein. The expression of Cas9 tagged with GFP in N27 cells is shown in Figure 4D. This lentiviral-mediated expression of the CRISPR-Cas9 system induced a drastic reduction of PK2 mRNA in N27 cells, as analyzed using qPCR (Figure 4E), and protein level as assessed by western blot assay using antibody against PK2 (Figure 4F). PK2 knockdown exacerbated mitochondrial damage induced by Mn treatment. Seahorse Mito-stress test of Mn-treated CRISPR-Cas9-
mediated PK2 knockdown or control knockdown in N27 cells (Figure 4G) shows lower oxygen consumption rate in PK2 knockdown cells (Figure 4H) and lower basal oxygen consumption rate (Figure 4I), as well as lower ATP production (Figure 4J). Compared to vector cells, PK2 knockdown N27 cells also exhibit a stressed phenotype (Figure 4K). This set of experiments suggests that PK2 could preserve and maintain mitochondrial function even during Mn-induced cellular stress while PK2 downregulation aggravates Mn-induced stress.

**Figure 5. Subacute and subchronic Mn treatment in mice increase PK2 levels in SN but not striatum.** A biologically relevant dose of Mn in the form of MnCl$_2$ (30mg/kg body weight) or vehicle was given daily via oral gavage to C57B/6 mice for 10 days (Figure 5A). After the subacute treatment, the mice were sacrificed, and brains dissected. Western blot analysis showed that in the SN, PK2 level was induced significantly (Figure 5B) whereas in the striatum, PK2 level was consistent (Figure 5C). This suggests that PK2 upregulation is an early response to Mn-induced neurotoxicity, and that PK2 is differentially regulated in the SN and the striatum.

Next, to better observe PK2 expression, we obtained Swiss Webster transgenic mice that expressed eGFP driven by the PK2 gene promoter. Mn in the form of MnCl$_2$ (30mg/kg body weight) or vehicle were given daily via oral gavage to eGFP-PK2 Swiss Webster mice for 30 days (Figure 5D). Mn-treated mice exhibited decreased locomotor activity as recorded by VersaMax software (Figure 5E). Specifically, Mn-treated mice exhibited lower horizontal activity, vertical activity, and total distance (Figure 5F). Interestingly, we also observed decreased social discrimination in mice treated with Mn, using the sniff test (Figure 5G), which measured a decrease in average number of visits to a zone with opposite sex’s bedding in Mn-treated mice and average time spent in zone with opposite sex’s bedding (Figure 5H).
Post-mortem analysis of biochemical changes in mice treated with Mn showed increased dopamine and decreased dopamine metabolites Dopac and HVA in Mn-treated mice as measured by HPCL, suggesting of decreased turnover of dopamine and a dysregulation of the dopamine metabolic pathway (Figure I). EGFP expression as driven by PK2 expression was generally increased in brains of mice treated with Mn, as measured by fluorescence intensity of eGFP using in-vivo imaging (Figure 5J). However, following dissection of the brain, western blot analysis showed that PK2 level was found to be decreased in the striatum of mice treated with Mn, whereas increases in olfactory bulb (OB) and SN were seen (5K). This suggests that PK2 is also upregulated during subchronic exposure to Mn, and that PK2 is also differentially regulated in the SN and the striatum. This interesting result had led us to assess the mechanism of differential regulation of PK2 in the striatum. Since Mn could epigenetically alter gene expression in dopaminergic cells in culture (Gandhi et al., 2017) and expression of PD-related genes (Tarale et al., 2017), we sought to assess the methylation status of the PK2 gene promoter in the cortex and striatum in mice treated with vehicle or Mn. Mn treatment induced a lower ratio of methylated to unmethylated PCR products in cortex tissues, suggesting decreased methylation of PK2 promoter region in the cortex, presumably allowing for higher transcriptional factor access to promoter and transcription of associated PK2 gene (Figure 5L). In the striatum tissues, however, PK2 promoter methylation status remains relatively unchanged (Figure 5M). This suggests that the failure to increase PK2 expression in the striatum could be due to epigenetic changes induced by Mn treatment. This set of experiments showed that Mn could exert its toxic effects through downregulation of PK2 gene expression in the striatum by inducing methylation of the PK2 promoter region.
Discussion

Although chronic exposure to excessive Mn produces a neurotoxic insult to the basal ganglia neurotransmitter system that culminates in Mn-induced Parkinsonism, the cellular and molecular mechanisms leading to neurotoxicity, especially early neuronal response to Mn exposure, are largely unknown and deserves more attention.

The mechanism causing the selective accumulation of Mn in basal ganglia, or why pallidal neurons are selectively vulnerable to Mn intoxication, is unclear (Bouabid, Tinakoua, Lakhdar-Ghazal, & Benazzouz, 2016). The mechanism of Mn toxicity in inducing cellular apoptosis is also not completely clear, but have generally been attributed to Mn’s capacity to cause oxidative stress, disrupt mitochondrial functions, and induce aberrant epigenetic changes in the genome (Kitazawa et al., 2005b; Latchoumycandane et al., 2005; Tarale et al., 2017; D. Zhang, Kanthasamy, Anantharam, & Kanthasamy, 2011). Mn could also exert neuronal toxicity by impairing key signaling molecules that control the balance between pro- and anti-apoptotic signaling. Although various proapoptotic signaling pathways that occur during neurotoxic stress have been elucidated, only a few studies have characterized signaling molecules that can protect neurons against toxic insults.

Recently, we reported that the prokineticin-2 (PK2) protein, a recently discovered mammalian homolog of mamba snake venom, plays a major compensatory protective function in nigral dopaminergic neurons. Here we found that the dopaminergic neurotoxicant Mn rapidly induce PK2 during the early stages of Mn neurotoxic stress in an N27 dopaminergic cell model. We showed that PK2 is a compensatory, protective response against Mn toxicity, and that overexpression of PK2 could protect against Mn-induced cell death. In light of Mn’s role in mitochondrial dysfunction, we showed that overexpression of PK2 protects against Mn-induced mitochondrial dysfunction while knockout of PK2 exacerbates these effects.
Importantly, we found that PK2 is differentially regulated in the striatum in relation to other regions experiencing less degeneration during Mn toxicity, likely to be caused by Mn-induced hypermethylation of PK2 promoter region in the striatum.

While pathophysiological differences exist in the diagnosis of PD and manganism, the underlying cellular mechanism of toxicity are overlapping. Mn mimics the action of MPP⁺ in selectively inhibiting mitochondrial complex I in neurons (Gavin et al., 1999; Hirata, Kiuchi, & Nagatsu, 2001; Malecki, 2001) by accumulating via mitochondrial Ca²⁺ uniporter (S. Zhang, Zhou, & Fu, 2003) to induce caspase 3 and 9 activation, major contributors to the execution of neuronal apoptosis leading to cell death (Choi et al., 2007; Smith, Fernandes, Go, & Jones, 2017; Uchida, Oh-Hashi, Kiuchi, & Hirata, 2012; L. Zhang, Sang, Liu, & Li, 2012). BCL-2, an anti-apoptotic protein localized on the outer mitochondrial membrane, could suppress caspase-3 activation induced by Mn toxicity (Anantharam et al., 2002; Latchoumycandane et al., 2005), DNA fragmentation, and PKCδ activation during neurodegeneration in cell culture models of PD (Fabisiak, Kagan, Ritov, Johnson, & Lazo, 1997; Kitazawa, Anantharam, & Kanthasamy, 2001; Yang et al., 1998). Therefore, in PD models, PK2-mediated upregulation of Bcl-2 could promote anti-apoptotic effects in dopaminergic neurons during toxic insult (Gordon et al., 2012; A. G. Kanthasamy et al., 2003; Kitazawa et al., 2005a, 2004). Our current study suggests that PK2 could preserve and maintain mitochondrial function even during Mn-induced cellular stress, while PK2 downregulation renders the cell susceptible to Mn-induced stress. In this study, we found downregulation of PK2 in striatum, pointing to a possible mechanism of selective vulnerability to Mn intoxication.

The preferential accumulation of Mn in the globus pallidus and striatum likely will also affect the subventricular zone (SVZ) along the lateral ventricle of the striatum, a major site of
neurogenesis in the adult brain. A recent study found that Mn caused an initial increase in early stages of neurogenesis in the SVZ, but due to Mn-induced toxicity, only 33% of new SVZ-derived neuroprogenitor cells (NPCs) survive to differentiate into functional neurons, compared to 64% in controls (Bresjanac & Antauer, 2000). Mn also exacerbates decreased neurogenesis and neuronal differentiation in the SVZ of MitoPark mouse model of PD (Langley et al., 2018). PK2 is a downstream target of Mash1 (C. Zhang et al., 2007) during neuronal differentiation as NPCs mature into neurons, a reduction of PK2 in the basal ganglia could therefore cause an accumulation of NPCs at the early stages of neurogenesis, and a reduction of NPCs undergoing neuronal differentiation in the SVZ to replace lost functional neurons in the striatum. Recent findings suggest that BDNF, an important trophic factor involved in neurogenesis (Bath, Akins, & Lee, 2012; Liu & Nusslock, 2018), was decreased in the striatum of non-human primates and mice chronically exposed to Mn, as well as primary cortical and hippocampal neuron cultures treated with Mn (Stansfield, Bichell, Bowman, & Guilarte, 2014). This raises the possibility that BDNF and PK2, which are both involved in olfactory bulb neurogenesis (Uranagase, Katsunuma, Doi, & Nibu, 2012; Yuan, 2008), are co-regulated in the long term in the striatum. It is also possible that the initial increase in neurogenesis is a compensatory response to acute Mn overexposure. If the exposure continues, however, the compensatory response is likely to be exhausted, with resultant decreases in neurogenesis in SVZ and cell loss in basal ganglia.

Some studies found that brain dopamine levels are initially elevated and then depleted by Mn toxicity (Cotzias, Miller, Papavasiliou, & Tang, 1976), suggesting that an initial compensatory response in the nigrostriatal system being overwhelmed. In our study, PK2 was upregulated in the SN after a subacute (10 days) Mn treatment, at a time during which no motor
impairments were detected. In the striatum, we saw an initial stabilization of PK2 level which later decreased. However, further studies are needed to clarify the role of PK2 in response against acute Mn exposure in striatum and the rest of the brain.

Current understanding of Mn toxicity in the brain attributes it on a function of its distribution; its preferential accumulation in the striatum causes damage to striatum while sparing the SN. However, upregulation of PK2 during early Mn neurotoxicity suggests that toxicity is not only limited to striatum, and might suggest PK2’s compensatory, protective role. PK2 upregulation in many regions relatively spared by Mn-induced toxicity contrasts with its downregulation in striatum, suggesting that the striatum did not participate in this compensatory response.

In light of the mechanism of Mn toxicity by way of interference with mitochondrial energy production, and given PK2’s role in mitochondrial biogenesis, failure to upregulate PK2 expression in the striatum due to Mn-induced PK2 promoter methylation could represent a major mechanism of toxicity, as well as a point of intervention by methods to upregulate PK2 expression in the affected regions.

Given that mitochondrial dysfunction has been increasing recognized as a central theme in other neurodegenerative disease aside from manganism and PD, such as in Alzheimer’s and ALS (Cozzolino, Ferri, Valle, & Carri, 2013; Maruszak & Żekanowski, 2011; Von Lewinski & Keller, 2005; Yu et al., 2018), understanding the role of PK2 in Mn toxicity could shed insight on mitochondrial dysfunction for a multitude of neurodegenerative diseases. Furthermore, current standard of care for manganism is limited to devising ways of reducing Mn by dietary intake, chelation therapy with metal chelators, and symptomatic management of dystonia (Kwakye, Paoliello, Mukhopadhyay, Bowman, & Aschner, 2015; Peres et al.,
2016; Rodan LH, Hauptman M, D’Gama AM, Qualls AE, Cao S, Tuschl K, Al-Jasmi F, Hertecant J, Hayflick SJ, Wessling-Resnick M, Yang ET, Berry GT, Gropman A, Woolf AD, 2018). Since a large portion of small molecules target a GPCR, PK2’s two cognate GPCRs, PKR1 and PKR2, represent clinically relevant druggable targets for pharmacological modulation of PK2 signaling using recombinant protein or chemical agonists.

In summary, exposure to Parkinsonian toxicants MPP+ and Mn induces PK2 upregulation in dopaminergic neuronal cells. Functional studies show over-expressing PK2 protect manganese-induced cytotoxic and apoptotic cell death. Mechanistic studies show that in PK2-overexpressing cells, pro-survival factors as well as factors important for mitochondrial health are preserved relative to vector control cells during neurotoxic insult. Manganese increases PK2 levels in the brain of GENSAT Swiss Webster mice, and decreases in the striatum. Overall, these results indicate that PK2 signaling represents a potential therapeutic target against Mn-induced neurotoxicity.

Materials and Methods

Animal handling. All animal procedures were approved by Iowa State University’s Institutional Animal Care and Use Committee (IACUC). All mice were housed under a 12-h light cycle in a climate-controlled mouse facility (22±1 °C) with food and water available ad libitum. Male C57B/6 mice were pre-screened during behavioral assessments for normal baseline performance before being randomly assigned to experimental groups. Animals were not wounded or otherwise exhibited conditions that could affect behavior, and are taken off the study if any conditions have manifested which could affect behavioral measurements. Investigators involved with data collection and analysis were not blinded to group allocation.
Behavior monitoring. All groups were monitored for behavior using the automated VersaMax system software connected to motion-detection hardware (VersaMax monitor, model RXYZCM-16, and analyzer, model VMAUSB, AccuScan, Columbus, OH). Each animal was put in one chamber fitted with motion-detection hardware, and allowed to acclimate for 2 minutes, after which its spontaneous locomotor activities (horizontal activity, vertical activity, and speed) were recorded for 10 minutes.

Western blot. Dissected brain regions were collected in Eppendorf tubes and flash frozen. To isolate total protein, RIPA buffer with sodium orthovanadate, Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher 78440) was added to each tube, and homogenized using a tissue homogenizer. Dissolved total lysate was centrifuged at 12,000g for 60 minutes, to remove cellular debris. Normalized protein samples were loaded into each well and were separated by sodium dodecyl sulfate (SDS) gel electrophoresis (100V, 90 minutes), using AnykD Mini-PROTEAN resolving gel (Bio-Rad, #4569035). Proteins on SDS gels were then transferred to a nitrocellulose membrane (26V, overnight) and blocked for 1h using fluorescent western blocking buffer (Rockland Immunochemicals). Primary antibodies were diluted in blocking buffer with 0.05% Tween 20, were then added to the membranes and incubated overnight at 4 °C. Next day, primary antibodies were taken off, and blots were washed (7 times, 5 minute each) in wash buffer comprising PBS containing 0.05% Tween 20 (PBST). Secondary antibody (infrared dye-tagged) was added for 1 h. Blots were further washed in PBST for 3 more times, and once in PBS. β-Actin was used as a loading control. Membranes were scanned using the Odyssey IR imaging system (LI-COR) and digital images were captured via LI-COR Odyssey imager. Densitometric analysis was done using ImageJ software.
**Immunohistochemistry.** Cells were fixed in 4% paraformaldehyde blocked with BSA and Triton-X to permeabilize the cell membranes. Primary antibodies were used as follows: rabbit anti-TH (1:1600 dilution), rabbit anti-PK2 (1:500 dilution), goat anti-GDNF (1:500 dilution), goat anti-IBA1 (1:1000), rabbit anti-GFAP (1:1000 dilution). Alexa Fluor Fluorescent Secondary Antibodies from ThermoFisher were used against primary antibodies for fluorescent images. For DAB staining, HRP-conjugated secondary antibodies were used against primary antibodies, and VECTASTAIN Elite ABC HRP Kit (VectorLabs) was used for conjugation of HRP to secondary antibodies. DAB was used for color development.

**SYBR Green qRT-PCR.** To obtain total RNA, tissue lysis buffer from Absolutely RNA Miniprep kit (Agilent Technologies) was added to dissected brain regions with addition of beta-mercaptoethanol as reducing agent to preserve RNA. A tissue homogenizer was used to dissolve tissue into the lysis buffer, and lysates were processed according to kit manufacturer’s instructions. Isolated total RNA from each sample was quantified using Nanodrop instrument to determine RNA concentration and purity. First strand cDNA synthesis was performed using Affinity Script qPCR cDNA synthesis system (Agilent Technologies) with 1µg of total RNA in the reaction mixture. Real-time PCR was performed with the RT² SYBR Green master mix (Qiagen) using diluted cDNA, and qPCR mouse primer sets were purchased from Qiagen (Quantitect primer mix). The 18S rRNA gene (mouse) was used as normalization of each sample as the housekeeping gene. The amount of each template was optimized empirically to maximize efficiency without inhibiting the PCR reaction. After the last cycle of the qPCR run, dissociation curves were run to ensure a single amplicon peak was obtained, indicating primer specificity. The results are reported as fold change in gene expression with the ΔΔCt method, using the threshold cycle (Ct) value for the housekeeping
gene and for the respective gene of interest in each sample. Control animals serve as the baseline for fold change.

**Lentivirus production.** PK2 lentivirus expression vector was obtained from Origene, and the MISSION Lentiviral Packaging Mix was obtained from Sigma (SHP001) and used according to manufacturer’s protocols. Briefly, the PK2 lentivirus expression vector was mixed with the Packaging Mix, and co-transfected into 293FT cells to package the virus. After 24h post-transfection, the supernatant, which contain the virus, was collected. The second harvesting of virus was done 48h post-transfection. The Lenti-X™ p24 Rapid Titer Kit from Clontech (632200) was used to titer the lentivirus.

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Figure 1. Neurotoxicant manganese treatment induces PK2 upregulation in N27 dopaminergic cells. A. Upregulation of PK2 mRNA with neurotoxicant Mn, MPP\(^+\) at 300\(\mu\)M, as well as HIF1\(\alpha\) stabilizer, DHB at 200\(\mu\)M, following their treatments for 4h. At the end of
the treatment, cell pellets were collected and processed for RNA isolation using Trizol. B. Upregulation of PK2 protein levels with Mn treatment time course of baseline, 6h, 8h, 10h, and 12h. At the end of treatment, cell pellets were collected and processed for western blot (top). Anti-PK2 and anti-β-actin were used to probe for PK2 and β-actin levels, and the results are analyzed using ImageJ software (bottom). Early upregulation of PK2 levels were seen at 6h post-treatment, and is sustained to 12h. Experiments were repeated twice with n = 3. Data represented as mean +/- s.e.m. and asterisks denote a significant (*P<0.05 and **P<0.01 and ***P<0.005) difference between control and treatment groups using one-way ANOVA with Tukey post-test comparison.
Figure 2. Stable PK2 overexpression in dopaminergic cell line protects against Mn-induced cytotoxic cell death. A. MN9D mouse dopaminergic cell line was transfected with PK2 overexpression vector by stable transfection of plasmid vector carrying human PK2
cDNA and a small myc-tag. After transfection, cells were put in growth media supplemented with neomycin/G418 to kill cells without transgene expression. Western blot runs for human PK2 indicates high expression in of PK2 in MN9D dopaminergic cells after stable transfection. B. Densitometric quantification of western blot analysis from A using ImageJ after normalization with beta-actin. Experiments were repeated three times. Data represented as mean +/- s.e.m. and asterisks denote a significant (*P<0.05 and **P<0.01 and ***P<0.005) difference between control and treatment groups using one-way ANOVA with Tukey post-test comparison. C. Localization of PK2, Myc-tag was determined by fluorescent immunostaining. Nuclei was stained by Hoechst dye (blue). Images were taken using 60x lens. E. MTS assay of Mn-treated Vector and PK2 overexpression cells, with control levels as percent control. Cells were treated with increasing doses of Mn ranging from 0µM to 500µM which were added to growth media with reduced serum (2% FBS) for 24 hours. F. Similarly, MTS assay of Mn-treated Vector and PK2 overexpression cells, but with addition of PC-7, a GPCR blocker that could block PKR2. G. Flowcytometry analysis of vector and PK2 cells treated with control or Mn. ANNEXIN-5 and propidium Iodide stains were used to determine percentage of cells that are in live, early apoptotic, late apoptotic, or dead stages 24h after Mn treatment. H. statistical analysis of data obtained from the flowcytometry experiment. Experiments were repeated twice with n = 3. Data represented as mean +/- s.e.m. and asterisks denote a significant (*P<0.05 and **P<0.01 and ***P<0.005) difference between control and treatment groups using one-way ANOVA with Tukey post-test comparison.
Figure 3. Stable PK2 overexpression in dopaminergic MN9D cells protects against Mn by reducing caspase activation and increasing BCL-2 levels. A. Caspase assay of vector and PK2 cells treated with either control or Mn for 16h. After treatment, cell pellets were collected
and processed for caspase assay, using Ac-DEVD-AMC caspase-3 as a fluorogenic substrate to assess caspase-3 activity. B. RT-PCR assay for Bcl-2 mRNA expression in vector and PK2 cells, treated with either control or Mn for 24h, with 18S as internal standard for normalization. C. Western blot for BCL-2 in Vector and PK2 cells treated with control, MPP+, or Mn for 8h. Experiments were repeated twice with n = 3. Data represented as mean +/- s.e.m. and asterisks denote a significant (*P<0.05) difference between control and treatment groups using one-way ANOVA with Tukey post-test comparison.
Figure 4. PK2 overexpression protects MN9D cells against Mn-mediated mitochondrial dysfunction, while knockdown of PK2 exacerbates Mn-induced mitochondrial dysfunction. 

A. Mitochondrial staining using MitoTracker dye staining for vector and PK2 cells treated with Mn for 24h revealed that mitochondrial staining were increased in PK2 cells.

B. RT-PCR analysis of mitochondrial DNA content (mDNA). Vector and PK2 cells are treated with control or Mn, and after 24h of treatment, cell pellets were processed. RT-PCR using
primers specific for mDNA cytochrome B revealed increased mDNA content in PK2 cells. C. Western blot analysis of mitochondrial levels of PGC1alpha and TFAM in vector and PK2 cells after treatment with control or Mn.

D. Activity of the CRISPR-Cas9 protein was assessed by green fluorescence, and the expression of Cas9 tagged with GFP in N27 indicates successful transduction of lentivirus in N27 cells. E. RT-PCR using rat PK2 primers found significantly reduced PK2 mRNA in PK2 knockout cells. F. Western blot analysis of PK2 protein level indicated significant reduction in PK2 protein in PK2 knockout cells. G. Seahorse Mito-stress test of CRISPR-Cas9-mediated PK2 knockdown in N27 cells. Vector cells and PK2 Knockdown cells were plated in Seahorse assay cell culture plate, and treated with control or Mn for 24h. H. CRISPR-Cas9-mediated PK2 knockdown in N27 cells shows lower oxygen consumption rate. I, J. PK2 knockdown N27 cells have lower basal oxygen consumption rate and lower ATP production. K. Compared to vector cells, PK2 knockdown N27 cells also exhibit a stressed phenotype. Experiments were repeated twice with n = 3. Data represented as mean +/- s.e.m. and asterisks denote a significant (*P<0.05) difference between control and treatment groups using one-way ANOVA with Tukey post-test comparison.
A. Mn 30mg/kg, daily

Behavioral at Day 1 and 10

B. Substantia nigra

C. Basal ganglia
D

Mn 30mg/kg, daily

Day 1  Day 30

Behavioral at Day 1 and 30

E

Control  Mn

F

Horizontal Activity  Vertical Activity  Total Distance

G

Control  Mn

Animal 1 (Test 1)  Mn  Animal 7 (Test 7)

H

Zone 1 = self bedding  Zone 2 = opposite sex bedding

Mean Visits to Zone 2  Time Spent in Zone 2

Average number of times  Seconds
Figure 5. Subacute and subchronic Mn treatment in mice increase PK2 levels in SN but not basal ganglia. A. Schematic of treatment paradigm for C57B/6 mice given Mn in the form of MnCl₂ (30mg/kg body weight) or vehicle, daily via oral gavage for 10 days. B, C. Western blot analysis for PK2 protein level in the SN and striatum. Wildtype C57B/6 mice brains were dissected after treatment and processed into lysates. Antibodies for PK2 were used to probe for PK2 protein level and beta-actin was used a loading control. PK2 levels were significantly higher after Mn treatment in the SN, but did not change in the striatum. D. Treatment paradigm for control and Mn treatment. Mn, in the form of MnCl₂ (30mg/kg body weight) or vehicle,
were given daily via oral gavage to eGFP-PK2 Swiss Webster mice for 30 days. **E.** A representative VersaPlot tracing of locomotor activity on open-field generated by VersaMax software. Mn-treated mice exhibited decreased locomotor activity. **F.** Mn-treated mice exhibited lower horizontal activity, vertical activity, and total distance traveled on open field. **G.** Representative heat map of tracked location of rodent’s head, generated by AnyMaze software. The social discrimination test involved planting bedding from opposite-sex on one end the of the long rectangular cage (zone 2), and rodent’s own bedding on the other end (zone 1). The number of times and the amount of time each mouse visits zone 1 or zone 2 and stays in the zone is recorded. **H.** Quantification of number of visits to zone 1 and zone 2 in mice in control or Mn treatment groups. **I.** HPLC analysis of dopamine, as well as dopamine metabolites DOPAC and HVA. After treatment course, the mice were sacrificed and brains dissected. The striatum was processed for HPLC. Increased dopamine and decreased metabolites were found, suggesting of decreased turnover of dopamine and a dysregulation of the dopamine metabolic pathway. **J.** Kodak in-vivo imaging revealed EGFP expression as driven by PK2 expression. After treatment, mice were sacrificed and whole brains were taken for imaging. **K.** Western blot analysis of PK2 protein level. PK2 level was found to be decreased in the striatum of mice treated with Mn, whereas increases in olfactory bulb (OB) and SN were seen. **L.** Methylation status of PK2 promoter region in the cortex after control or Mn treatments. Methylation-specific PCR was performed on cortex samples from mice treated with control or Mn and run on a agrose gel. M = methylated PCR product, U = unmethylated PCR product. After Mn treatment, a lower ratio of methylated to unmethylated PCR products were seen, presumably allowing for higher transcriptional factor access to promoter and
transcription of associated genes. **M. PK2** promoter methylation remains relatively unchanged in the striatum.
CHAPTER 4. PHARMACOLOGICAL MODULATION OF ASTROCYTE-
DERIVED GDNF BY PROKINETICIN 2 RECEPTOR AGONIST IS20:
PRECLINICAL ASSESSMENTS USING MPTP AND MITOPARK RODENT
MODELS OF PARKINSON’S DISEASE

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Abstract

Despite a wealth of preclinical studies establishing neuroprotective and
neurorestorative properties of glial cell-line derived neurotrophic factor (GDNF) in animal
models of PD, a number of phase II clinical trials utilizing direct intracranial injection of GDNF
protein and AAV-mediated Gdnf gene transfer did not achieve efficacy that was hoped for.
Setbacks from recent clinical trials might prompt a rethinking of the strategy which focused on
ectopic expression of GDNF targeted towards neurons. Devising strategies to elevate GDNF
expression by means other than genetic manipulation is the current challenge. We have
previously found that prokineticin signaling is a compensatory protective response against
dopaminergic neuronal degeneration during PD. Here we show that GDNF has significant
crosstalk with prokineticin signaling in astrocytes. A small molecule, IS20, could activate
prokineticin signaling to induce secretion and increased gene expression of GDNF from
astrocytes in culture via activation of prokineticin receptor 1 (PKR1) preferentially expressed
by astrocytes. Further, noninvasive administration of the blood-brain-barrier-permeable

lipophilic IS20 through intranasal delivery could pharmacologically modulate GDNF levels in brain and in the nigrostriatal system in C57B/6 mice. Importantly, IS20 treatment yielded significant neuroprotective and neurorestorative effects in MPTP-induced and MitoPark genetic mouse models of PD. Our results indicate that the full clinical benefit of GDNF could be leveraged by pharmacological modulation using IS20.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease that affects around 1% of the world's aging population. This disease is characterized by progressive degeneration of dopaminergic neurons of the substantia nigra pars compacta (SNpc) in the ventral midbrain, leading to subsequent dopamine loss within the striatum, resulting in severe motor impairment that includes akinesia, rigidity, and tremor (Barzilai & Melamed, 2003; Jarraya et al., 2009). Since the 1950s following the Nobel-winning discovery of the role of dopamine precursor 3,4-dihydroxy-L-phenylalanine (L-DOPA) in Parkinsonian animals by Swedish scientist Arvid Carlsson, the current standard-of-care treatment strategy for PD has continued to be the oral administration of L-dopa, which increases the availability and concentration of dopamine in the striatum to alleviate motor symptoms, but has little effects on nonmotor symptoms (Meissner et al., 2011; W & O, 1961). Since no disease-modifying therapy has been developed (Won et al., 2016), the progressive nature of the disease demands dosage titration involving high doses of L-dopa, resulting in an array of motor fluctuations and other severe complications including dyskinesia over time in the majority of patients (Jarraya et al., 2009).

Glial-cell derived neurotrophic factor (GDNF) has been well documented to participate in the development and maintenance of various neuronal systems in the body, including
development of neuronal system in the kidney, enteric nervous system (ENS), and the central nervous system (CNS). Since its discovery in 1992 (O’Malley, Sieber, Black, & Dreyfus, 1992), GDNF has been one of the most potent neurotrophic factors discovered to date with protective properties on the dopaminergic system (L. F. Lin, Doherty, Lile, Bektesh, & Collins, 1993). Dopaminergic neurons express both GDNF receptor GFRα1 and GDNF co-receptor RET, through which GDNF promotes regeneration and survival of SN dopaminergic neurons. GDNF was also found to increase dopamine content and uptake, while stimulating neurite extension, and increase in tyrosine hydroxylase (TH) in cell culture models (Beck et al., 1995; L. F. Lin et al., 1993). In vivo studies provide evidence that GDNF is necessary for maintenance of dopaminergic neurons during later stages of development and throughout adulthood. Supporting this notion, conditional knockout of GDNF in mice starting at 1 month of age results in selective decrease of TH immunoreactivity and a 60-70% loss of DA neurons in midbrain at 7 months of age, further indicating that under normal conditions, GDNF is indispensable for DA neuron survival (Pascual et al., 2008).

Numerous preclinical studies using rodent and primate models of PD has demonstrated the potential of GDNF as a treatment for PD (Bilang-Bleuel et al., 1997; Björklund, Rosenblad, Winkler, & Kirik, 1997; J. H. Kordower et al., 2000; Tseng, Baetge, Zurn, & Aebischer, 1997) However, initial clinical trials delivering recombinant GDNF protein using intracranial administration failed to diffuse into deeper layers of the brain and slow down neurodegeneration or improve motor function (Domanskyi, Saarma, & Airavaara, 2015). While recent improvements in GDNF delivery has shown moderate efficacy (Gill et al., 2003), it still does not overcome the difficult challenge of long-term GDNF delivery into the brain. GDNF gene therapy using AAV-mediated gene delivery can overcome the hurdle of targeted
delivery, and was proven safe in phase I clinical trial, but ultimately showed no efficacy in phase II (Blits & Petry, 2016; Remy, 2014; Tenenbaum & Humbert-Claude, 2017). Furthermore, in the current configuration, AAV-mediated gene delivery is irreversibly integrated into genome of target cells, and is thus inherently less controllable than classical pharmacotherapies (Tereshchenko, Maddalena, Bähr, & Kügler, 2014). Failures of current GDNF trials underscore the importance of comprehending the events upstream of GDNF signaling pathway and deconvoluting other signaling pathways in crosstalk with GDNF. The control of endogenous GDNF expression would constitute a powerful target in neuropharmacology (Caumont, Octave, & Hermans, 2006). Devising strategies to elevate its expression by means other than genetic manipulation is the current challenge that should warrant increased translational research efforts (Ibáñez & Andressoo, 2017).

Setbacks of current AAV-mediated dopaminergic GDNF gene delivery trials might demand a rethinking of the strategy, including expressing GDNF in striatal astrocytes. Astrocytes secrete neurotrophic factors NGF, BDNF, and GDNF (Dougherty, Dreyfus, & Black, 2000), and are the major source of GDNF in brains of PD patients (Drinkut, Tereshchenko, Schulz, Bähr, & Kügler, 2012; Mogi et al., 2001). Astrocyte’s role in maintaining a healthy environment for proper functioning of neurons has been increasingly recognized, yet their potential as a source of nigral GNDF and as therapeutic targets has not been sufficiently leveraged.

Initially identified from a nontoxic component of black mamba snake venom (Laustsen, Lomonte, Lohse, Fernández, & Gutiérrez, 2015), the Prokineticins are shown to crosstalk extensively with GDNF signaling pathway in the peripheral nervous system during development of ENS (Enomoto et al., 1998; Ngan et al., 2007, 2008; Young et al., 2001), and
defects in either signaling pathways produce the rare genetic condition Hirschsprung disease (Ruiz-Ferrer et al., 2011). GDNF and prokineticin crosstalk during the development of ENS raises the possibility that the prokineticin signaling pathway is also in similar crosstalk with GDNF in the CNS. Interestingly, both GDNF and PK2 have been shown to be strong chemoattractants for axons during neurogenesis/neuritogenesis, and PK2 is required for complete olfactory bulb (OB) development. Importantly, we previously found that PK2 upregulation mediates a protective, compensatory response through activation of AKT, ERK pathway and increasing mitochondria biogenesis during neurodegeneration (Gordon et al., 2016). AAV2/5-mediated delivery of PK2 could protect against neurodegeneration induced by Parkinsonian toxicants in the SN and striatum. However, until now, there is no evidence for a fundamental relationship between PK2/PKR1 and GDNF/RET signaling in the CNS and the nigrostriatal system. Intriguingly, PKR1 was also found to be mainly expressed in astrocytes, raising the possibility that GDNF could be co-upregulated with the prokineticin pathway in brain astrocytes. Moreover, the predominant expression of PKR1 compared to PKR2 in astrocytes(Koyama et al., 2006) suggests that astrocytes could be preferentially targeted using receptor agonists with specificity to PKR1. Thus, using a lipophilic small molecule receptor agonist specific to PKR1 (Gasser et al., 2015), IS20, we hypothesized that positive modulation of the expression and secretion of GDNF in astrocytes of the nigrostriatal system can be achieved via pharmacological activation of PK2/PKR1 signaling. Subsequently, this could promote trophic effects on the dopaminergic system to yield neuroprotective and neurorestorative effects in PD models.

In the present study, we report that 1) PK2/PKR1 signaling represents a novel, druggable pathway that could modulate GDNF expression in the CNS, particularly in the
astrocytes, 2) the upregulation of GDNF through PK2/PKR1 signaling could be pharmacologically induced by intranasal delivery of the PKR1 receptor chemical agonist IS20, and 3) IS20 could rescue DA neurons and neuronal projections against neurodegeneration in genetic and chemically-induced mouse models of PD. In contrast to long-term uncontrolled continuous expression driven by intracerebral injections of GDNF viral vectors, which led to unwanted compensatory responses (B. Georgievska, 2004; Biljana Georgievska, Kirik, & Björklund, 2002; Sajadi, Bauer, Thöny, & Aebischer, 2005), intranasal IS20 administration showed no such responses, nor any overt toxicity in mice for the course of treatment. Therefore, by pharmacologically modulating the amount and duration of endogenous GDNF expression using small molecule IS20 treatment, GDNF’s established clinical benefits could be fully harnessed with non-invasive procedures and minimum unwanted compensatory responses.

Results

Figure 1. Prokineticin receptor 1 is activated by recombinant PK2 protein and receptor agonist IS20. To examine whether the PK2 agonist IS20 can positively modulate GDNF expression, our initial set of experiments was conducted to confirm that IS20 is able to activate PKR1, similar to recombinant PK2 (rPK2) protein. PKR1 has been reported to be expressed on the membrane of dopaminergic neurons and type 1 astrocytes in the nigrostriatal system. Once activated, PKR1 initiates downstream signaling activation that culminates in the rise of Ca^{2+} in the cytosol (Ngan & Tam, 2008). To facilitate the analysis of PKR1 activation, we used a CHO cell line stably expressing PKR1. The PKR1-overexpressing cells were treated with 10 µM IS20 or 100 nM rPK2, and PKR1 activation was monitored by measuring intracellular Ca^{2+} mobilization in real time over 180 seconds using a Fluo-4 NW calcium assay kit. In silico modeling of binding to PKR1 protein reveals that both rPK2 (Fig. 1A) and IS20
(Fig. 1C) can bind to PKR1 with high affinity. As shown in Fig. 1B, upon treatment with rPK2, intracellular Ca\(^{2+}\) concentration rapidly rises and peaks at 20 seconds post-treatment, suggestive of a G\(\alpha_q\)1 mechanism of PKR1 activation (Fig. 1B). Similarly, after treatment with 10 \(\mu\)M of IS20, Ca\(^{2+}\) concentration rises but slowly peaks at 180 seconds post-treatment (Fig. 1D), suggesting that IS20 induces a similar magnitude but relatively delayed activation of PKR1 compared to rPK2.

**Figure 2. PK2 agonist IS20 and recombinant PK2 upregulate GDNF expression in U373 human astrocyte cells.** Next, to examine the effects of PK2 signaling activation on GDNF expression in astrocytes we first treated U373 human astrocyte cells with rPK2 and found that treatment with 25 nM rPK2 was able to induce GDNF protein expression to 1.6-fold above control (Fig. 2A), and mRNA level to nearly 3-fold above control (Fig. 2B). The rPK2-induced upregulation of GDNF mRNA was completely attenuated with co-treatment of the Prokineticin receptor antagonist PC-7 (Fig. 2B). Furthermore, using an ELISA kit, we showed that the release of GDNF in media was significantly increased by rPK2 (25 nM). To confirm these findings, we infected U373 cells with lentivirus expressing PK2-GFP fusion protein or GFP control virus. Although we only achieved a modest increase in PK2 levels (Fig. 2D), Lenti-PK2-GFP-infected cells exhibited a 1.7-fold increase in PK2 protein levels as compared to Lenti-GFP-infected control cells (Fig. 2E). Importantly, time-course treatment of U373 cells with 10 \(\mu\)M IS20 induced maximal GDNF mRNA expression at 1 h; this upregulation declined over the subsequent 5 h (Fig. 2G). To assess any receptor-specific effects of IS20 on GDNF production, we overexpressed PKR1 by transiently transfecting increasing amounts of PKR1 expression vector (0.5-15 \(\mu\)g) in U373 astrocytes and the transfected cells were treated with 10\(\mu\)M IS20 for 4h. As shown in Fig. 2H, ectopic expression of PKR1 dose-
dependently stimulated IS20-induced upregulation of GDNF mRNA, and the optimal stimulation (5-fold) was saturated between 5-15 µg PKR1 expression plasmid. Next, we generated a stable PKR1 overexpression U373 cell line and found that PKR1 overexpression cells exhibited an increased expression of GDNF mRNA and immunoreactivity, and treatment with IS20 (3-10 µM) further potentiated this upregulation (Fig. 2I-J). More importantly, when a CRISPR-Cas9 guide RNA against PKR1 was used to knock down PKR1 (Fig. 2K) in U373 astrocytes, IS20 was no longer able to modulate GDNF expression level (Fig.2L), further demonstrating that IS20-induced GDNF expression is highly dependent on PKR1 receptor binding and activation.

Figure 3. IS20 protects against MPP⁺-induced cell death and preserves mitochondrial energetics in dopaminergic neuronal cells. Having discovered that IS20 is a potent inducer of GDNF in astrocytes, we further evaluated whether the IS20-induced upregulation and secretion of astrocyte-derived GDNF could functionally protect dopaminergic neuronal cells under insults from the classic Parkinsonian toxicant MPP⁺. U373 astrocytes were incubated with or without IS20, and the astrocyte conditioned media (ACM) was collected and added to N27 dopaminergic neuronal cells, with co-treatment with or without MPP⁺ (100-300 µM) for 24 h. As shown in Fig. 3A, addition of IS20 ACM significantly increased the cell viability in MPP⁺-treated N27 cells compared to the control ACM/MPP⁺-treated cells. Next, we investigated whether this protection was mediated through preservation of mitochondrial respiration. Seahorse analysis revealed that in addition to slightly increasing the basal respiration of untreated N27 cells (Fig. 3B, red and blue bars), IS20 ACM remarkably attenuated MPP⁺-induced reduction in oxygen consumption rate, basal respiration, reserve capacity, and ATP production (Fig. 3B-E), suggesting a post-mitochondrial
mechanism at least partly contribute to the protection conferred by IS20 ACM. Lastly, we utilized mouse primary striatal neurons to validate the neuroprotective effects of IS20 ACM. We included the ACM collected from U373 astrocytes treated with IS21, a compound known not to activate PKR1, to further determine the specificity of IS20. As depicted in Fig. 3F, IS20 ACM but not IS21 ACM promoted the cell survival in MPP⁺-exposed mouse primary striatal neurons as determined by MTS assays. All together, these studies suggest that U373s treated with IS20 could secrete factors that protect dopaminergic neuronal cells against MPP⁺-induced neurotoxicity.

**Figure 4. IS20 upregulates GDNF expression in primary mouse astrocytes and midbrain organotypic slice cultures.** Next, we used cultured primary mouse astrocytes from prenatal mouse pups to validate the findings obtained from U373 astrocyte cells. A time-course study of the effects of 10 µM IS20 treatment on the expression of GDNF mRNA (Fig. 4A) and the release of GDNF protein (Fig. 4B) over a 8-h period revealed rapid upregulation of GDNF mRNA and release, most prominent at 2h and 3h post-treatment, respectively, which gradually declined over time back to the levels slightly above those of control groups. To further determine the effect of the concentration of IS20 on GDNF mRNA expression and protein release, we undertook a concentration response study using a range of 0.3-30 µM of IS20. As shown in Fig. 4C-D, the maximal response was achieved for an IS20 concentration of 10 µM on both GDNF mRNA expression and protein release. GDNF protein levels in the IS20-treated whole cell lysates also increased concomitantly in a time- and dose-dependent manner (Fig. 4E), demonstrating that GDNF is both upregulated and secreted by primary astrocyte cells following IS20 treatment. Supporting this, treatment of primary mouse astrocytes with 10µM of IS20 for 8 h can increase GDNF immunoreactivity in western blot (Fig. 4F). Some of the
primary mouse astrocytes that were isolated were separately stained for GFAP to confirm that they were indeed astrocytes (Fig. 4G).

Interestingly, mRNA expression of GDNF and its predominant cell surface receptor in the dopaminergic system, GFRα1, were co-upregulated 3 h post-10 μM IS20 treatment (Fig. 4G), indicating that the increased gene expression and protein secretion of GDNF also can lead to an upregulation of its receptor in the same cells, possibly establishing a positive feedback loop which helps to increase GDNF upregulation and secretion. To further evaluate the effect of IS20 on GDNF expression, we treated organotypic midbrain slice cultures prepared neonatal mouse pups. In line with the above findings, IS20 treatment for 4 h in cultured organotypic slices increased GDNF mRNA expression (Fig. 4H) and protein levels (Fig. 4I). Taken together, these results demonstrated that GDNF is highly upregulated and secreted in primary mouse astrocytes and midbrain organotypic slice cultures following IS20 treatment.

**Figure 5. IS20 administration induces GDNF expression and release in C57 black mice.** Next, we determined the bioavailability of IS20 in the brain after intraperitoneal injection or intranasal administration, and looked for its biological effects in the brain. Wildtype C57 black mice were intraperitoneally injected with DMSO or IS20 at 10 mg/kg body weight for 8 h, and GDNF protein levels in the brain and serum were examined by Western blot and ELSA, respectively. As shown in Fig. 5A-B, we observed a significant increase in GDNF protein levels in nigral lysates with a concurrent increase in serum. Intranasal administration allows for small, relatively lipophilic compounds intended for the CNS to non-invasively bypass much of the blood-brain-barrier (Hernando et al., 2017), thus increasing compound bioavailability in the CNS while reducing exposure to peripheral tissues. Given this information, in a second study, we therefore determined to examine the bioavailability of the lipophilic IS20 and its
effect on GDNF expression following intranasal administration of 3 mg/kg IS20. A lower dose (3 mg/kg) was chosen because intranasal administration has been shown to concentrate lipophilic compounds more efficiently in the brain than intraperitoneal injections. Using liquid chromatography/mass-spectrometry (LC/MS), our pharmacokinetic study found that the concentration of intranasally injected IS20 accumulated to be 4 ng/mg tissue in the brain at 30 minutes, gradually decreased to 1.5 ng/mg tissue at 90 minutes and to less than 0.8 ng/mg tissue at 6 h, indicating that intranasally injected IS20 was able to cross the blood brain barrier and accumulate in the brain. Interestingly, 24 h post IS20 treatment, the brain level of IS20 was highly maintained. Similar to intraperitoneal injection of IS20, at 3 and 6 h post-intranasal administration of IS20, qPCR assays showed significant increases in GDNF mRNA expression in SN and striatum (Fig. 5D). ELISA detected concurrent increases in GDNF protein in both brain lysates and serum (Fig. 5E), and the maximal response was observed at 30 minutes post IS20 treatment. GDNF mediates pro-survival effects through its preferred receptor GFRα1, a GPI, and its co-receptor RET, a receptor tyrosine kinase that transduces intracellular signals, and decreased expression of RET has been shown to cause progressive degeneration of the nigrostriatal system (Sariola, 2003; Tenenbaum & Humbert-Claude, 2017). Intriguingly, IS20 modestly but significantly increased the expression of GFRα1 and RET mRNAs in whole brain (Fig. 5F). Collectively, these data show that both minimally-invasive intraperitoneal delivery and non-invasive intranasal delivery of IS20 could upregulate GDNF and GDNF receptors in the mouse brain.

**Figure 6. IS20 protects against MPTP-induced dopaminergic cell death in C57 black mice and restores GDNF expression.** Since intracranial delivery of exogenous recombinant GDNF has shown effective neuroprotection (Wang, Lin, Chiou, Williams, &
Hoffer, 1997), we hypothesized that the early induction of GDNF in striatum induced by intraperitoneally administered IS20 was sufficient to protect dopaminergic neurons against chemical-induced neurodegeneration. To test this hypothesis, we intraperitoneally injected C57BL/6 mice with the classic Parkinsonian toxicant MPTP, followed by an intraperitoneal injection of IS20, each day for 5 days. Since we wish to evaluate the effect of IS20 during neurodegeneration and not as a preventative measure, IS20 was administered 1 h after MPTP administration. After 5 days of the MPTP and IS20 co-injections, we injected only IS20 for 7 more days (Fig. 6A). GDNF gene expression in the striatum was then analyzed by qPCR, which confirmed that MPTP treatment alone mildly reduced GDNF gene expression, and that IS20 co-treatment modestly but significantly restored GDNF gene expression in mice that received MPTP injections (Fig. 6B). Also, IS20 treatment alone upregulated GDNF gene expression in mice as anticipated in coherence with the above-mentioned in vitro and in vivo pharmacodynamics experiments (Fig. 6B). Importantly, HPLC analysis of striatum homogenates showed that IS20 treatment modestly but significantly attenuated the MPTP-induced depletion of dopamine and its metabolites HVA and DOPAC (Fig. 6C-E). Finally, to further determine the effects of IS20 on the lesioned nigral-striatal system and to confirm the functional effects of IS20-induced GDNF upregulation on the preservation of dopaminergic system, TH immunohistochemistry and stereological counts of TH-positive dopaminergic neurons were performed on caudate-putamen and substantia nigra (SN) cryosections obtained from mice in each group. As expected, MPTP significantly reduced the number of TH-positive dopaminergic neurons in the SN. However, IS20 co-treatment significantly restored TH-positive neuron count in MPTP-treated mice (Fig. 6F-G). TH immunohistochemistry analysis also showed that IS20 treatment significantly preserved dopaminergic nerve fibers damaged
by MPTP treatments (Fig. 6G, representative sections from each group shown). These results suggest that IS20 treatment is protective against MPTP-induced neurochemical deficits resulting from MPTP-induced dopaminergic neurotoxicity, possibly through enhanced GDNF signaling.

**Figure 7. IS20 protects against dopaminergic neurodegeneration in Mitopark PD model.** We next tested if intranasal administration of IS20 could restore behavioral and motor functions in the MitoPark mouse model. Starting at 15 weeks, MitoPark mice exhibited significant and drastic reduction in locomotor activity as shown by representative Versaplot maps (Fig. 7A) and slowed horizontal activity (Fig. 7B). However, after 14 days, MitoPark mice that received IS20 intranasal administration performed mildly better in horizontal activity compared to vehicle control, and by day 28 post treatment, MitoPark mice that received IS20 performed significantly better (Fig. 7B-C). Taken together, these sets of experiments showed that IS20 intranasal delivery could significantly protect against dopaminergic neuronal cell loss and behavioral deficits in the MitoPark genetic model of PD.

**Figure 8. IS20 protects against dopaminergic neurodegeneration in the Mitopark mouse model of PD.** Because dopaminergic degeneration in the MPTP model requires an acute neurotoxic insult and follows a rapid disease course, we next used the MitoPark mice, a chronically progressive neurodegenerative mouse model of PD to verify if intranasal administration of IS20 in a longer-term (4 week) treatment regimen protects against dopaminergic neurodegeneration in a more gradual and progressive model. The MitoPark mouse model is a genetic model of PD rendered by conditional knockout of mitochondrial transcription factor A (TFAM) driven by the Cre/Loxp system in dopaminergic neurons, which results in close recapitulation of PD symptoms, including a steady, progressive loss of
dopaminergic neurons over several months, with the accompanying loss of motor capacity starting at around age 12-14 weeks, as well as non-motor symptoms including olfactory deficits and depression. Mice born in the same litter but does not have conditional knockout of TFAM and DAT exhibited no deficits, and serve as ideal health controls. MitoPark mice aged 13 weeks, the average onset of motor symptoms, were randomly chosen for treatment with vehicle or IS20 for 4 weeks. Similar to mice treated with MPTP, GNDF gene and protein expression, as measured by qPCR and Western blot, respectively, in substantia nigra (SN) of MitoPark mice were significantly reduced compared to their litter mates (Fig. 8A-B). Importantly, treatment with IS20 significantly increased GDNF gene expression and protein levels in MitoPark mice (Fig. 8A-B). Moreover, TH protein levels in the (SN) of MitoPark mice were significantly restored after treatment with IS20 (Fig. 8B), whereas PKR1 levels were not affected. Since astrocyte-derived GDNF could reduce microglia-induced neuroinflammation (Chen et al., 2015; Rocha, Cristovão, Campos, Fonseca, & Baltazar, 2012), we also looked for microglia activation by immunostaining IBA1 in the SN MitoPark mice treated with vehicle or IS20. IBA1 immunoreactivity was significantly reduced in MitoPark mice by IS20 treatment (Fig. 8C), suggesting that IS20 treatment also prevents microglial activation in the SN of MitoPark mice. To confirm the functional effects of IS20-induced GDNF upregulation on dopaminergic neurodegeneration in MitoPark mice, brain sections from 16-wk MitoPark mice were immunostained with TH to detect dopaminergic neurons. Intranasal IS20 treatment significantly preserved the dopaminergic neuronal cell bodies in SN and dopaminergic neuronal fibers that projected into the striatum of MitoPark (Fig.8E-F). DAB immunostaining and stereological counts of TH-positive dopaminergic neurons further showed significantly
higher neuronal counts (Fig. 7G-H) in the SN of IS20-treated MitoPark mice compared to vehicle-treated MitoPark mice.

**Figure 9. IS20 activates p44/42, AKT pathways and induces pro-survival NRF2, BCL2 gene expression.** Since phosphorylation of AKT could induce GDNF upregulation, we sought to find effects of IS20 on cellular signal transduction in primary mouse astrocytes. IS20 rapidly induced AKT phosphorylation and p44/42 phosphorylation (Fig. 9A). Further, IS20 induced early expression of Nrf2 (Figure 9B) and Bcl2 (Fig. 9C). In C57B/6 mice, IS20 8h after IP injection induced AKT phosphorylation in striatum and heart, the organ in which the protective effects of IS20 was originally found (Figure 9D). These experiments suggest that mechanistically, IS20 could induce signaling transduction known to induce GDNF upregulation, and induce other neuroprotective factors.

**Discussion**

In this study, we demonstrated the potential for a small-molecule PKR1 receptor agonist to induce GDNF expression and to achieve neuroprotective and neurorestorative effects in two rodent models of PD. In particular, we showed that activation of PKR1 signaling through IS20 treatment quickly causes GDNF secretion and gene expression in cultured astrocytes and organotypic slices. In C57B/6 mice, intranasally administered IS20 successfully crosses the blood brain barrier to reach maximum concentration in around 30 minutes and induce GDNF serum release and gene expression in the nigrostriatal system. In a subacute MPTP mouse model of PD, IS20 protects dopaminergic neurons against MPTP-induced neurodegeneration and increases dopamine levels in the striatum of MPTP-treated mice. Furthermore, we showed that, in transgenic MitoPark mice, 4-week intranasal IS20 administration induces GDNF and TH levels in the SN, reduces microglial activation and
protects against dopaminergic neurodegeneration, and these protective effects translate to functional improvements in locomotor activity in MitoPark mice. These findings suggest that pharmacological modulation of GDNF signaling could be achieved with minimum side effects using intranasal administration of a PKR1 receptor agonist, IS20.

PK2 activates PKR1 at EC$_{50}$ 4.3 +/- 1.3 nM and PKR2 at EC$_{50}$ 7.3 +/- 2.8 nM (D. C.-H. Lin et al., 2002). The apparently similar receptor affinities (Zhou, 2006) suggest that downstream effects induced by PK2 are determined by the expression levels of PKR1 and PKR2 on cell surface (Attramadal, 2009). We previously have shown that PK2 signals through PKR1 and PKR2 in neurons to rapidly increase intracellular Ca$^{2+}$ and induce phosphorylation of AKT and p44/42, leading to increases in BCL2 protein levels and mitochondrial biogenesis (ref). We also developed an efficient AAV2/5-mediated gene delivery system, which produced robust, stable expression of PK2 in the striatum and protected substantia nigra neurons from MPTP-induced neurodegeneration. In astrocytes, activation of prokineticin signaling is likely modulated by the predominant expression of PKR1 (Koyama et al., 2006). Our current study shows that the PKR1 receptor agonist IS20 rapidly increases intracellular Ca$^{2+}$ and induces phosphorylation of AKT and p44/42 in astrocytes (Figure 10). Activation of AKT is a known pathway that results in secretion and upregulation of endogenous GDNF. Since both prokineticin and GDNF signaling depend on AKT and ERK signaling in the dopaminergic neurons to mediate protective effects (Gordon et al., 2016; Sariola, 2003), it is therefore possible that prokineticin and GDNF signaling crosstalk here to mediate IS20’s pro-survival effects. Indeed, blocking AKT and p44/42 using API and PD98059, respectively, in neurons treated with PK2 protein nullifies protective effects, while blocking p44/42 using PD98059 prevented GDNF release from rat glioma cells (Tanabe, Matsushima-Nishiwaki, Iida, Kozawa,
These data further suggest that prokineticin and GDNF signaling converge at AKT and p44/42 in dopaminergic neurons to promote neuronal survival.

Recently, it was revealed that GDNF is upregulated by ischemia (K Yamagata et al., 2002) and astrocyte-derived GDNF could protect neuronal cultures from cell death in cases of cellular mitochondrial damage (C.-H. Lin et al., 2006; K Yamagata et al., 2002). PK2 is also upregulated by hypoxia in primary cortical culture models of ischemia, induces proliferation, survival and migration of capillary endothelial cells in hypoxic stress, and supports mitochondrial biogenesis under MPP⁺-induced oxidative stress (Cheng et al., 2012; LeCouter et al., 2003). These previous findings support our hypothesis that PK2 is co-regulated with GDNF in the brain. Other reports also found that PK2 signaling has significant crosstalk with GDNF signaling in the development of the enteric nervous system (Ngan et al., 2008). Both PK2 and GDNF are strong chemoattractants for projecting axons during neurogenesis and neuritogenesis. Conditional knockout of PKR1 leads to heart and kidney disorders due to deficits in angiogenesis, cell pro-survival signaling, mitochondrial, and progenitor cell functions in these organs (Boulberdaa et al., 2011). GDNF and PK2 signaling in these cellular functions suggest a possible crosstalk between them in both peripheral nervous system and the central nervous system.

Previous GDNF clinical trials concluded that although the potential for GDNF-based therapy remains high, two challenges need to be overcome: proper selection of animal PD models during preclinical testing and unwanted compensatory reactions or side-effects associated with GDNF delivery (Taylor et al., 2013). MPTP is the most frequently used Parkinsonian toxicant applied in the generation of animal models of PD (Beal, 2001; Przedborski et al., 2001), with the obvious advantage that of MPTP was clinically observed in
1979 to produce a human model of the disease upon accidental injection (Davis et al., 1979; Langston, Ballard, Tetrud, & Irwin, 1983). However, although subacute MPTP treatment (3-5 daily injections) in rodents could manifest neuropathology in PD, it does not follow the progressive nature of the disease in humans or reliably reproduce behavioral deficits that recapitulate the human disease in rodents (Rommelfanger et al., 2007; Schober, 2004; Tillerson, Caudle, Reverón, & Miller, 2002). MPTP injections leave striatal dopaminergic neuron projections intact, capable of retrograde-transporting GDNF to neuron soma in the SN. Yet, recent clinical data observed in post-mortem brains of PD patients 5 years post-diagnosis revealed that TH-positive dopaminergic innervations from the caudate-putamen completely disappeared, questioning the effectiveness of delivering GDNF only in the striatum of advanced PD patients, as employed by clinical trials conducted by Amgen (Lang et al., 2006; Nutt et al., 2003; Tatarewicz et al., 2007) and others (Slevin et al., 2005, 2007). MitoPark mice used in the current study, in comparison, had similarly lost striatal projections at 17 weeks, recapitulating conditions seen in advanced PD patients, thereby allowing critical assessments of preclinical functional endpoints. Early studies that achieved neuroprotective effects indicated that GDNF administration by intranigral injection could mitigate neuronal cell loss, but did not protect against loss of projections (LeCouter et al., 2003; Mandel, Spratt, Snyder, & Leff, 1997). Intrastriatal injection of AAV-GDNF could protect against degeneration of both neuronal cell body and projections, provided that it is administered before neuronal cell loss (Kirik, Rosenblad, Bjorklund, & Mandel, 2000). This is presumably because to achieve full effectiveness, GDNF must be retrograde-transported to dopaminergic neuron soma for RET signaling to mediate pro-survival effects (Tenenbaum & Humbert-Claude, 2017). Results from past studies using only acute neurodegeneration models which suggested that targeting the
striatum was both necessary and sufficient are confounded when a lack of axon transport deficiencies in the model is taken into consideration (Herzog et al., 2013). Therefore, high GDNF expression in striatum is less effective if GDNF retrograde-transport is disrupted due to significantly degenerated dopaminergic neuronal projects. In this case, increasing GDNF level in both striatum and SN might prove to be more effective in mediating GDNF-induced pro-survival effects (Marks et al., 2010). Indeed, success have been achieved by using AAV2-mediated neurotrophic factor gene transfer in both striatum and nigra (Kirik et al., 2000; Jeffrey H. Kordower et al., 2006). In our current study, IS20-induced GDNF expression level was upregulated in both striatum and substantia nigra of MitoPark mice, allowing astrocyte-secreted GDNF to protect both dopaminergic projections in striatum as well as neuron soma in SN. Significantly more striatal projections are preserved in MitoPark mice treated with IS20 (Figure 9). Together, these data indicate that performing preclinical studies in animal models that recapitulate the progression of PD pathology could increase success rate of translational research for PD and reduce attrition of candidate therapies.

Most current GDNF gene delivery trials use AAV2, which has low immunogenicity and has proven safe for use in humans, but transduces predominantly neurons (Carter, 2001; Ruitenber, Eggers, Boer, & Verhaagen, 2002; Towne, Pertin, Beggah, Aebischer, & Decosterd, 2009). However, in the injured brain, GDNF production is mainly regulated by astrocytes and not by neuronal cells (Bresjanac & Antauer, 2000; Nakagawa & Schwartz, 2004). Astrocytes therefore provide the major source of GDNF in the substantia nigra of PD patients’ brains (Drinkut et al., 2012; Mogi et al., 2001) and secrete GDNF during ischemia as a compensatory responses (Kazuo Yamagata et al., 2007). Astrocytes also secrete other neurotrophic factors CDNF, BDNF, NGF, and in the midbrain, astrocytes and microglia
outnumber neurons 10 to 1 (Azevedo et al., 2009; Rocha et al., 2012). The major source of GDNF in the gut is also from glia cells, the enteric glia cells (Steinkamp et al., 2012; Von Boyen et al., 2011). Early studies isolated GDNF from type 1 astrocytes from the SN and found that astrocyte-derived GDNF enhanced dopaminergic neuron survival when nigral astrocytes were co-cultured with dopaminergic neurons as support cell monolayers (O’Malley et al., 1992). Therefore, neuroprotective efforts targeting only neurons for long-term neuronal viability are unlikely to succeed if supportive astrocytes do not provide proper neurotrophic and metabolic enrichments (Takano, Oberheim, Cotrina, & Nedergaard, 2009). Several studies have found that intrastriatal viral-mediated GDNF gene delivery causes aberrant neurite growth towards the site of application (Biljana Georgievaska et al., 2002; Tenenbaum & Humbert-Claude, 2017), suggesting that mimicking endogenous mode of expression is important to achieve functional effects. Several studies had focused on viral-delivery of GDNF into astrocytes, achieving localized, yet satisfactory neuroprotection (Drinkut et al., 2012). Building upon these studies, we found here that targeted GDNF upregulation in astrocytes could be achieved pharmacologically using non-invasive route of administration, which suggests that more research efforts should be placed back to astrocytes.

The neuroprotective and neurorestorative effects afforded by IS20 could also be mediated through reduction of IBA1 expression in the SN. Astrocytes’ role in neuroinflammation associated with neurodegeneration has been increasingly recognized. SN has an extremely high density of microglia (Ouchi et al., 2005), and activated microglia are a common feature in this area, which is affected by PD pathology (Rickert et al., 2014). GDNF secreted from astrocytes has been shown to potently inhibit excessive production of reactive oxygen species from microglia in zymosan A-stimulated midbrain microglia cultures (Rocha
et al., 2012) and LPS-stimulated primary midbrain neuron-glia cultures (Wu et al., 2009). It is therefore also possible that astrocyte-derived GDNF could mediate protective effects by its anti-inflammatory effects on activated microglia (Rickert et al., 2014). In line with previous studies, we showed that IS20 treatment reduced IBA1 expression in the SN of 17-week MitoPark mice (Figure 9B). Furthermore, constitutive GDNF expression from viral transgene expression can cause downregulation of TH expression (Tereshchenko et al., 2014), possibly due to feedback loops between GDNF and dopamine reuptake (Gomes, Vaz, Ribeiro, & Sebastião, 2006; Kopra et al., 2017). Instead, a discontinuous GDNF delivery paradigm, in which GDNF levels were allowed to return to basal level, showed a reduction in such unwanted compensatory effects (Taylor et al., 2013; Tereshchenko et al., 2014). In the current study, pharmacodynamics studies in healthy control mice found that IS20-induced GDNF expression in striatum, SN, as well as whole brain returned to basal levels 6 h post-treatment (Fig. 5), avoiding constitutive GDNF expression. Consequently, we saw increases in TH in MitoPark mice treated with IS20 (Fig. 8). Yet another possible cause of unwanted compensatory effect results from high GDNF expression, at least one or two orders of the magnitude higher, driven by viral transgenes in other studies (Ibáñez & Andressoo, 2017). In our study, we have seen 2-fold and 3-fold increase in GDNF expression induced by IS20 in the striatum and SN, respectively, and 0.5-fold increase over the control level in GDNF expression in whole brain (Fig. 5E). Further, we did not see overt signs of toxicity or significant reduction in bodyweight with administered doses and routes of administration while the mice were on study. This is in agreement with a previous published study, whereby subchronic intraperitoneal administrations of PK2 protein, which activates PKR1 and PKR2 in a relatively non-selective
fashion (Lattanzi et al., 2012), was administered systemically in C57Bl/6 mice and resulted in no serious toxicity seen (Beale et al., 2013).

In summary, IS20 administration could pharmacologically upregulates GDNF levels in the nigrostriatal pathway, preserving dopaminergic neurons and functional innervations in neuroprotective and neurorestorative paradigms applied in PD animal models. The relative safety profile of IS20 makes it a promising pharmacological candidate for PD therapy.

Materials and Methods

Calcium Assays. Intracellular calcium mobilization was assessed using Fluo-4 NW Calcium Assay Kit (F36206) according to manufacturer’s instructions. Briefly, CHO cells were grown on 96 well plates the previous day. Molecular Devices FLIPR instrument was used to inject dissolved IS20 into the cell plate.

Animal handling. All animal procedures were approved by Iowa State University’s Institutional Animal Care and Use Committee (IACUC). All mice were housed under a 12-h light cycle in a climate-controlled mouse facility (22±1 °C) with food and water available ad libitum. Male C57B/6 mice were pre-screened during behavioral assessments for normal baseline performance before being randomly assigned to experimental groups. Animals were not wounded or otherwise exhibited conditions that could affect behavior, and are taken off the study if any conditions have manifested which could affect behavioral measurements. Investigators involved with data collection and analysis were not blinded to group allocation.

MPTP injections. Over a 5-day period, one i.p. injection of 18 mg kg MPTP was administered every day to each mouse in the MPTP group, and an equal volume of saline (vehicle) is received by the saline group.
Behavior monitoring. All groups were monitored for behavior using the automated VersaMax system software connected to motion-detection hardware (VersaMax monitor, model RXYZCM-16, and analyzer, model VMAUSB, AccuScan, Columbus, OH). Each animal was put in one chamber fitted with motion-detection hardware, and allowed to acclimate for 2 minutes, after which its spontaneous locomotor activities (horizontal activity, vertical activity, and speed) were recorded for 10 minutes.

MitoPark transgenic mice. MitoPark transgenic mice were a kind gift from Dr Nils-Göran Larsson of Karolinska Institute, Stockholm, from his laboratory at the Max Planck Institute for Biology of Ageing. MitoPark mouse model is created by inactivation of mitochondrial transcription factor A (Tfam) specifically in DA neurons by conditional knockout through control of the dopamine transporter promoter. The mice used were from the MitoPark breeding colony at Iowa State University. All mice were housed under a 12-h light cycle in a climate-controlled mouse facility (22±1 C) with food and water available ad libitum. After behavioral experiments were performed at the indicated ages, mice were sacrificed using procedures approved by Iowa State University’s Institutional Animal Care and Use Committee (IACUC). Mice were euthanized at the indicated time points via carbon dioxide asphyxiation as outlined in approved animal use protocols, and samples processed for either qRT-PCR or western blotting.

Western blot. Dissected brain regions were collected in Eppendorf tubes and flash frozen. To isolate total protein, RIPA buffer with sodium orthovanadate, Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher 78440) was added to each tube, and homogenized using a tissue homogenizer. Dissolved total lysate was centrifuged at 12,000g for 60 minutes, to remove cellular debris. Normalized protein samples were loaded into each
well and were separated by sodium dodecyl sulfate (SDS) gel electrophoresis (100V, 90 minutes), using AnykD Mini-PROTEAN resolving gel (Bio-Rad, #4569035). Proteins on SDS gels were then transferred to a nitrocellulose membrane (26V, overnight) and blocked for 1h using fluorescent western blocking buffer (Rockland Immunochemicals). Primary antibodies were diluted in blocking buffer with 0.05% Tween 20, were then added to the membranes and incubated overnight at 4 °C. Next day, primary antibodies were taken off, and blots were washed (7 times, 5 minute each) in wash buffer comprising PBS containing 0.05% Tween 20 (PBST). Secondary antibody (infrared dye-tagged) was added for 1 h. Blots were further washed in PBST for 3 more times, and once in PBS. β-Actin was used as a loading control. Membranes were scanned using the Odyssey IR imaging system (LI-COR) and digital images were captured via LI-COR Odyssey imager. Densitometric analysis was done using ImageJ software.

**Immunohistochemistry.** Cells were fixed in 4% paraformaldehyde blocked with BSA and Triton-X to permeabilize the cell membranes. Primary antibodies were used as follows: rabbit anti-TH (1:1600 dilution), rabbit anti-PK2 (1:500 dilution), goat anti-GDNF (1:500 dilution), goat anti-IBA1 (1:1000), rabbit anti-GFAP (1:1000 dilution). Alexa Fluor Fluorescent Secondary Antibodies from ThermoFisher were used against primary antibodies for fluorescent images. For DAB staining, HRP-conjugated secondary antibodies were used against primary antibodies, and VECTASTAIN Elite ABC HRP Kit (VectorLabs) was used for conjugation of HRP to secondary antibodies. DAB was used for color development.

**SYBR Green qRT-PCR.** To obtain total RNA, tissue lysis buffer from Absolutely RNA Miniprep kit (Agilent Technologies) was added to dissected brain regions with addition of beta-mercaptoethanol as reducing agent to preserve RNA. A tissue homogenizer was used.
to dissolve tissue into the lysis buffer, and lysates were processed according to kit manufacturer’s instructions. Isolated total RNA from each sample was quantified using Nanodrop instrument to determine RNA concentration and purity. First strand cDNA synthesis was performed using Affinity Script qPCR cDNA synthesis system (Agilent Technologies) with 1μg of total RNA in the reaction mixture. Real-time PCR was performed with the RT² SYBR Green master mix (Qiagen) using diluted cDNA, and qPCR mouse primer sets were purchased from Qiagen (Quantitect primer mix). The 18S rRNA gene (mouse) was used as normalization of each sample as the housekeeping gene. The amount of each template was optimized empirically to maximize efficiency without inhibiting the PCR reaction. After the last cycle of the qPCR run, dissociation curves were run to ensure a single amplicon peak was obtained, indicating primer specificity. The results are reported as fold change in gene expression with the ΔΔCt method, using the threshold cycle (Ct) value for the housekeeping gene and for the respective gene of interest in each sample. Control animals serve as the baseline for fold change.

Lentivirus production. PK2 lentivirus expression vector was obtained from Origene, and the MISSION Lentiviral Packaging Mix was obtained from Sigma (SHP001) and used according to manufacturer’s protocols. Briefly, the PK2 lentivirus expression vector was mixed with the Packaging Mix, and co-transfected into 293FT cells to package the virus. After 24h post-transfection, the supernatant, which contain the virus, was collected. The second harvesting of virus was done 48h post-transfection. The Lenti-X™ p24 Rapid Titer Kit from Clontech (632200) was used to titer the lentivirus.

HPLC analysis of striatal dopamine levels. HPLC samples were prepared and processed as previously described (Gordon et al., 2016). Briefly, mice were euthanized, striata
were collected and neurotransmitters were extracted in 0.2 M perchloric acid solution containing 0.05% Na₂EDTA, 0.1% Na₂S₂O₅ and isoproterenol (internal standard). Dopamine and metabolites were separated isocratically by a reversed-phase column with a flow rate of 0.6 ml min⁻¹ using a Dionex Ultimate 3000 HPLC system (pump ISO-3100SD, Thermo Scientific, Bannockburn, IL) equipped with a refrigerated automatic sampler (model WPS-3000TSL). The electrochemical detection system included 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.

References


Figure 1. Prokineticin receptor 1 is activated by recombinant PK2 protein and receptor agonist IS20. A. Model of PK2 binding to PKR1. B. PK2 then induces intracellular Ca$^{2+}$ mobilization as measured by Fluor4NW assay kit on FlexStation. C. The lipophilic small molecule IS20 could bind inside the binding pocket of PKR1. D. The receptor agonist IS20 could induce intracellular Ca$^{2+}$ mobilization as measured by Fluor4NW assay kit.
Figure 2. PK2 and PK2 agonist IS20 increase GDNF in U373 human astrocyte cell line.

A. Recombinant PK2 protein was dissolved in reduced serum (2% FBS) and incubated on U373 human astrocyte cells. Western blot assay found increased GDNF level in U373 treated
with 25nM PK2 recombinant protein, B. qPCR assay found increased GDNF gene expression in cells treated with 25nM PK2, but not when PC-7, a PKR1/PKR2 blocker, was co-incubated with IS20. C. GDNF level in U373 cell lysates was also found to increase in GDNF ELISA assay. D, E. Lentiviral-mediated delivery of PK2 cDNA into U373 cells induced stable expression of PK2-eGFP as shown by fluorescent microscopy and western blot. F. GDNF level was found to increase in U373 transduced by lentiviral PK2-eGFP by western blot assay. G. qPCR assay found that treatment with PKR1 agonist IS20 could induce GDNF gene expression level in a time dependent manner. H. Quantitative PCR analysis of GDNF gene expression.

overexpressed PKR1 expression vector is transiently transfected in increasing amounts of cDNA (0.5-15 µg) in U373 astrocytes, and the transfected cells were treated with 10µM IS20 for 4h. Overexpression of PKR1 vector dose-dependently stimulated IS20-induced upregulation of GDNF mRNA, and the optimal stimulation (5-fold) was saturated between 5-15 µg PKR1 expression plasmid. I. Stable PKR1 overexpression U373 cell line and found that PKR1 overexpression cells exhibited slightly increased expression of GDNF mRNA. J. PKR1 overexpression cells seems to have higher GDNF immunoreactivity, and treatment with IS20 (3-10 µM) further potentiated this upregulation. K. Quantitative PCR showed that CRISPR-Cas9 guide RNA against PKR1 produced efficient knock down of PKR1 in U373 astrocytes. L. Quantitative PCR assay showed that IS20 was no longer able to modulate GDNF expression in PKR1 knock out cells. NTC: nontarget control.
Figure 3. IS20 protects against MPP⁺-induced cell death and preserves mitochondrial energetics in dopaminergic neuronal cells. A. MTS assay of N27 dopaminergic neurons after co-treatment with MPP⁺ and astrocytes conditioned media (ACM). To make Control ACM and IS20 ACM, U373 astrocytes were treated with or without IS20 for 2 h followed by a change
to fresh media and an additional incubation for 6 h. This astrocyte conditioned media (ACM) was collected and added to N27 dopaminergic neuronal cells, together with no MPP⁺ or MPP⁺ (100-300 µM) for 24 h. B. Seahorse analysis of N27 cells treated with: Control ACM (blue line), IS20 ACM (red line), IS20 ACM added with MPP⁺, and MPP⁺ alone. B. IS20 ACM remarkably attenuated MPP⁺-induced reduction in oxygen consumption rate, C. basal respiration, D. reserve capacity, and E. ATP production F. MTS assay of primary striatal neurons isolated from the mouse. The same Control ACM, Control ACM added with MPP⁺, IS20 ACM + MPP⁺, IS21 ACM + MPP⁺, and the proper controls, were used to treat primary striatal neurons.
Figure 4. IS20 increases GDNF in primary mouse astrocytes in culture. **A.** GDNF ELISA found increased GDNF level in supernatant of primary mouse astrocytes treated with 10µM IS20 at 2h over a time course of 1h to 8h. **B.** The optimum time point (2h) was chosen for dose response of IS20 on GDNF secretion. At 2h, ELISA found significantly increased GDNF secretion by primary mouse astrocytes treated with 10µM IS20. **C.** Similarly, qPCR found increased GDNF gene expression in primary mouse astrocytes treated with 3µM IS20 at 3h and 4h over a time course of 1h to 7h. **D.** The optimum time of 3h was chosen for a dose response study. At 3h, qPCR assay found greatest increase in GDNF gene expression with 10µM IS20 treatment. **E.** At 3h, qPCR analysis of GDNF receptor GFRα1 gene expression also showed increase at 10µM IS20. **F.** Western blot analysis of primary astrocytes treated with 1µM, 10µM IS20 or 25nM PK2 revealed increase in IS20 protein level in primary mouse astrocyte cultures at 4h and 8h post-treatment (top). Densitometric analysis of western blot shows approximately 2-fold increase in GDNF protein level (bottom). **G.** Immunostaining of GFAP confirms astrocytic marker in cultured primary mouse astrocytes. **H.** Quantitative PCR analysis of PK2 gene expression in cultured midbrain organotypic slices treated with 0, 3, 30µM of IS20 for 4h. **I.** Western blot analysis of PK2 protein levels in cultured organotypic slices treated with 0 or 30µM of IS20 for 4h.
Figure 5. Intraperitoneal or intranasal administration of IS20 induces GDNF family neurotrophic factors in naïve C57 black mice. A. Western blot analysis showed that intraperitoneal injection of 10mg/kg IS20 increased levels of GDNF 8 h post-administration. B. GDNF in blood serum was also found by ELISA to increase at 8h. C. Liquid-chromatography/mass spectrometry (LC/MS) analysis showed that intranasally administered IS20 become concentrated to approximately 4ng/mg in the brain at 30 minutes post-
administration and rapidly reduced to less than 1 ng/mg at 6h in a time course from 30 minutes to 24h. D. ELISA assay showed highest GDNF level in brain (left) and blood serum (right) 30 minutes post-administration which slowly reduced to basal levels at 24h. E. Quantitive PCR showed that At 3h post-intranasal administration, GDNF expression was increased in whole brain, SN, and striatum, while at 6h post-administration, only SN still had a modest but significant increase in GDNF gene expression level. F. Quantitive PCR also showed that gene expression of GDNF receptor GFRα1, and co-receptor RET both were increased 3h post-administration of S20.
Figure 6. IS20 protects against MPTP-induced dopaminergic cell death in C57B/6 mice and restores GDNF expression. A. Treatment paradigm for MPTP and IS20 treatments. C57B/6 mice was peritoneally-injected with MPTP daily for 5 days. IS20 (10mg/kg) was injected peritoneally daily following MPTP injections, and continued for 7 more days after
cessation of MPTP injections, for a total of 12 injections total. B. After 12 days of treatments, GDNF gene expression was assessed by qPCR which found significant increases in striatum of the group co-treated with IS20 compared to MPTP group. HPLC analysis showed drastic decreases in dopamine (C), dopamine metabolites homovanillic acid (D) and DOPAC (E), in MPTP-treated group, while IS20 co-treatment group showed significantly increased levels of dopamine and metabolites compared to MPTP group. F. Software assisted dopaminergic neuron count of each group showed significantly more TH+ dopaminergic neurons in SNpc of IS20 co-treated mice compared to MPTP group. G. DAB immunohisto-staining of TH+ neurons in SNpc shown as 2x magnification (top) and 10x images (bottom).
Figure 7. IS20 protects against behavioral deficits in MitoPark model. A. Treatment paradigm of IS20 3mg/kg via intranasal administration. MitoPark mice were treated with
vehicle or IS20 every other day for 28 days. Weekly behavioral monitoring using B. VersaMax apparatus and software showed movement tracing over 10 minutes of monitoring C. Activity data from VersaMax showed restoration of horizontal locomotor activity after 28 days of treatments with IS20.
Figure 8. IS20 protects against dopaminergic neurodegeneration in MitoPark model.

A. Mitopark mice (13-wk-old) were subjected to IS20 intranasal delivery, 3mg/kg every other day for 28 days. Quantitative PCR showed that IS20 treatment increased GDNF gene
expression in SN, N = 6. Western blot analysis of SN brain lysates probed using antibodies against GDNF, TH, IBA1, PKR1, showed increased levels of GDNF, TH, decreased IBA1, while PKR1 stayed constant. B. β-Actin was used as loading control. N = 6. C. Densitometric analysis of western blot analysis shown in B. Student’s T test, P value < 0.05 (*), < 0.01 (**), < 0.01 (**). D. SN brain sections co-immunostained with IBA1 and fluorojade showed decreased fluorojade immunoreactivity in MitoPark mice treated with IS20. E. Tyrosine hydroxylase (TH) immunostaining of SN sections. Images were taken at 2X magnification (top) and 10X magnification (bottom). F. Computer-assisted stereological counting of TH+ cells present in the SNpc. G. SN brain sections co-immunostained with TH and fluorojade in the SN showed increased TH immunoreactivity and decreased fluorojade staining in the group that received IS20.
Figure 9. IS20 activates p44/42, AKT pathways and induces pro-survival NRF2, BCL2 gene expression. 

A. Western blot analysis of phosphorylated AKT, as well as p44 and p42. IS20 rapidly induced AKT phosphorylation and p44/42 phosphorylation. Primary astrocytes were treated and collected at 0.5h, 1h, 6h post-treatment with IS20. 

B. Quantitative PCR showed that IS20 induced a trend of early expression of Nrf2, although not statistically significant. 

C. Quantitative PCR showed that induction of Bcl2 gene expression was
significantly higher 1h post-treatment with IS20, and steadily decreased towards control levels at 2h and 3h. D. Western blot analysis of phospho-AKT and AKT in C57B/6 mice. 8h after IP injection, mice were sacrificed and probed for AKT phosphorylation in striatum and heart.
A

Healthy Control

PD Patient

B

GDNF mRNA expression in Human PD patients
Supplementary Figure 1. A. Double-labelling immunofluorescence for GDNF (red), PKR1 (green) in nigral sections from PD patients and age-matched controls probed using antibodies directed against the human GDNF and PKR1 proteins. Decreased levels of GDNF were evident in PD patient samples compared with control subjects while PKR1 levels seemed constant. Scale bar, 50 μm. For both Healthy Control panel and PD patient panel, the top portion is ×60 magnification to show PKR1 merging with several PKR1-positive cells and the bottom portion is with ×2.5 zoom to get a high magnification image of PKR1 and GDNF in one cell. B. Quantitative PCR showing GDNF downregulation in striatum of PD patients. Data represented as mean±s.e.m. and expressed as fold change over control with n=10. Asterisks denote a significant (***P<0.01) difference between control and PD samples using Student’s t-test.
CHAPTER 5. CONCLUSIONS AND FUTURE DIRECTIONS

This section serves as a general summary of the research chapters presented previously, and to discuss possible directions for future work on this topic. In this work, we showed that during MPP⁺⁻-induced neurodegeneration, NRF1, EGR1 and HIF1α are potential regulators of PK2 gene expression. We then demonstrated the mechanism of PK2-mediated protection on Mn-treated dopaminergic neuronal cell culture model of PD via mitochondrial biogenesis and BCL-2 upregulation, and showed its in vivo relevance in Mn mouse model of PD. We lastly demonstrated the clinical relevance and potential of PK2 signaling via pharmacological activation of GDNF in MPTP and MitoPark model of PD.

The decade-long neurodegenerative process is complex, involving toxicant exposure creating oxidative stress and mitochondrial dysfunction, exacerbated by self-propagating neuroinflammation. Little is yet known about epigenetic alternation, particularly DNA methylation, by environmental toxicants, such as Mn. Altered expression of PD-related genes such as alpha-synuclein, and PK2, by epigenetic mechanisms illustrate the process of environmental-gene interactions, and could account for differences in individual susceptibility and variability through the course of disease progression. This represents a new area of research that deserves more focus.

PK2 Transcriptional Regulation during Neurodegeneration

Despite recent advances in understanding of PK2’s functions in the brain, its role and its transcriptional regulation during neurotoxic stress remain incompletely understood. It has been shown that in the CNS, PK2 can be transcriptionally regulated via Enhancer-box (E-box) binding by basic helix-loop-helix (bHLH) transcription factors. CLOCK/BMAL1, the
circadian rhythm master regulators, and Ngn1/MASH1, proneural genes expressed during olfactory bulb neurogenesis, have been determined to regulate PK2 transcription. Further, HIF1α, another bHLH transcription factor, is speculated to regulate PK2 transcription via binding to several putative HIF1α binding sites on the PK2 proximal promoter. Our studies confirmed that overexpressed HIF1α could induce PK2 promoter activity and gene expression, while also confirming the involvement of EGR1 in regulating PK2 in a similar manner. Importantly, we found that except in the striatum, PK2 expression generally co-regulated with EGR1 and HIF1α in Mn overexposure model of neurotoxic stress. Further mechanistic studies conducted in dopaminergic cell cultures using CRISPR cas9-based knockdown and chemical inhibition of HIF1α, demonstrated that EGR1 and HIF1α are major regulators of PK2 gene expression during MPP⁺-induced PK2 upregulation. It has been reported that treatment with DHB, a HIF1α stabilizer, could rescue mitochondrial dysfunction, a major mechanism of DHB-mediated protective effects (D. W. Lee et al., 2009). Since PK2 could induce proliferator-activated receptor-γ coactivator 1α (PGC-1α) in the activation of mitochondrial biogenesis and respiration (Gordon et al., 2016), it therefore could be speculated that HIF1α activation of PK2 could help in mediating PGC-1α’s protective effects. Further studies need to be conducted to confirm the mechanism of a putative HIF1α-PK2-PGC-1α axis.

EGR1 activation of PK2 transcription leading to neurotrophic factor upregulation represents another potential direction for future work. EGR1 is activated by MAPK/ERK signaling pathway to stimulate neurite outgrowth in a traumatic brain injury model (Chasseigneaux et al., 2011; Plummer, Van den Heuvel, Thornton, Corrigan, & Cappai, 2016). EGR1, EGR2, EGR3, and EGR4 are co-upregulated with brain-derived neurotrophic factor (BDNF) after treatment with pridopidine in Huntington disease mouse models (Kusko et al.,
EGR1, EGR2, EGR4 expression are induced by erythropoietin (EPO), a neuroprotective cytokine in models of ischemic injury (Mengozzi et al., 2012), while PK2 is also co-upregulated during ischemic injury (M Y Cheng et al., 2012; Landucci et al., 2016). Further, EGR1 could induce angiogenesis via induction of PK1 in the peripheries (Sheng et al., 2018). In light of our current findings that suggest GDNF induction by PK2, further efforts could be put on confirming a putative EGR1-PK2-AKT-PI3K-GDNF signaling axis.

An alternative pathway could also be proposed. EGR1 could also activate high levels of GDNF by directly binding to the Gdnf promoter (Shin et al., 2009). It has also been shown that PK2 could reciprocally activate EGR1 expression via ERK1/2 activation. Together with our findings currently presented here, this represent a reciprocal, positive-feedback loop that induces expression of both PK2 and EGR1. Therefore PK2-ERK-EGR1-GDNF signaling could represent an alternative signaling axis that deserves future effort for elucidation and validation. The contribution from each putative signaling pathway would also need to be evaluated.

We have also found, in our present studies, that PK2 expression is induced early during neurodegenerative process induced by Mn overexposure, suggesting that PK2 could serve as a potential marker neurodegeneration. Elucidating the transcriptional mechanism underlying PK2 upregulation in dopaminergic neurons allow us to identify events upstream and players involved in PK2 upregulation, and begin to find other similarly co-regulated pathways. Understanding the perturbations in these complex pathways due to environmental factors or neurodegenerative events is crucial for targeting any pathway, such as the PK2 signaling pathway, to achieve neuroprotection.
Neuroprotective Effects of PK2 signaling

We had previously reported that PK2 is induced during neurodegeneration in multiple cell culture, animal model, and clinical cases of PD. These data revealed a perhaps new function of PK2 in the brain, especially in relevance to PD. Following logical next steps, we had also reported the effects of AAV-mediated PK2 gene delivery in the striatum, a concept similar to delivery of AAV-neurturin in the mouse striatum (Jeffrey H. Kordower et al., 2006; Marks et al., 2010). We reported protection against MitoPark and MPTP-induced neurodegeneration and reduced inflammatory microglial activation in mice treated with AAV-PK2 (Gordon et al., 2016). However, due to requirements of AAV-based gene delivery to be stereotaxically injected into the striatum, and the possibility of tumorigenesis as seen in the AAV-neurturin studies, which had found tumors in both mice injected with AAV vector as well as AAV-neurturin, long-term gene delivery via AAV is not currently optimal.

Therefore, in the studies presented here, we showed the potential for a small-molecule PKR1 receptor agonist to activate PKR1 signaling and induce GDNF expression in cultured astrocytes and organotypic slices. Treatment with IS20 demonstrated neuroprotective and neurorestorative effects, suggesting its clinical relevance in PD therapy. In C57B/6 mice, intranasally administered IS20 successfully crosses the blood brain barrier to reach maximum concentration in around 30 minutes. Using C57B/6 mice injected with MPTP as mouse model of PD, we had found IS20 to protect dopaminergic neurons against MPTP-induced neurodegeneration and increases dopamine levels in the striatum of MPTP-treated mice. Furthermore, we showed that, in transgenic MitoPark mice, 4-week intranasal IS20 administration induces GDNF and TH levels in the SN, reduces microglial activation and protects against dopaminergic neurodegeneration. These protective effects translate to functional improvements in locomotor activity in MitoPark mice. These findings also suggest
that pharmacological modulation of GDNF signaling could be achieved with minimum side effects using intranasal administration of a PKR1 receptor agonist, IS20. Furthermore, no exogenous proteins are injected, nor does IS20 persist in the brain. For the above reasons, pharmacological modulation of endogenous GDNF levels that could be tightly controlled might resolve issues associated with protein diffusion, leakage, and transgene tumorigenicity.

Further considerations and experimentation are needed to improve and optimize the efficacy of intranasal administration of IS20. Optimization of formulations for carrier solutions, such as pH, choice of buffer solutions, and total volume could drastically improve delivery of IS20 into the nasal cavity and bioavailability in the CNS. Diffusion models that take into account the drug transport through the paracellular space across the nasal epithelium and perineural space, to reach the subarachnoid space of the brain, could immensely aid in design of delivery methods as well as determination of optimal dosing regimen (Cowie et al., 2017).

**Induction of Neurotrophic Factors by PK2**

Current undergoing work is examining the effect of IS20 on other family members of the GDNF family, neurturin, artemin, and persephin, in cell culture models. Even though artemin co-receptor GFRα3 and persephin co-receptor GFRα4 have limited expression in the adult brain, neurturin and co-receptor GFRα2 are constitutively expressed in the adult brain, and neurturin has shown to approximate the neuroprotective effects demonstrated by GDNF. Since neurturin is being evaluated in phase II clinical trials, potential induction of neurturin by IS20 represents a new opportunity for study.

Our preliminary studies suggest that PK2 could also induce GDNF in bone marrow-derived stem cells, imply that IS20, as a PKR1 ligand, could also induce GDNF in these cells.
Given the therapeutic potential of stem cells in PD therapy for their capacity to secrete neurotrophic factors, PKR1-overexpressing stem cells that are implanted in PD patients could exhibit heightened production of neurotrophic factors upon IS20 treatment. Therefore, IS20-induced GDNF upregulation in other cell types aside from astrocytes, if confirmed, could suggest larger, farther-reaching effects of IS20 in the brain.

**Further Considerations: Use of Appropriate Animal Models of PD**

It is worthwhile to emphasize that proper models of disease are needed to reduce pipeline attrition from a drug development perspective. Administration of Parkinsonian toxicant in rodents and primates has been widely employed for elicitation of dopaminergic neuronal cell death and PD-like motor symptoms. In preclinical studies for PD, they have been used in evaluating therapies that mitigate motor symptoms. However, in the narrow effort to obtain motor deficits and subsequently mitigate these motor symptoms using candidate therapies, less attention has been paid to assessing the animal models’ ability to recapitulate the chronic and progressive nature of the disease, or the specific processes associated with dopaminergic neurodegeneration. For example, subacute MPTP treatment (3-5 daily injections) in mice is widely used to generate loss of dopaminergic cells in SNpc, but it does not follow the progressive nature of the disease in humans, and due to rodents’ higher capacity for compensatory response against dopamine loss, do not reliably reproduce behavioral deficits (Rommelfanger et al., 2007; Schober, 2004; Tillerson, Caudle, Reverón, & Miller, 2002). Importantly, MPTP treatments do not induce complete loss of striatal dopaminergic neuron projections, while clinical data from observation of PD patient post-mortem brains showed complete degeneration of dopaminergic projections in the caudate-putamen at 5 years post-diagnosis. Therefore, while putamenal gene delivery of GDNF can induce substantial GDNF
expression overall in the brain, the anticipated beneficial effects from anteriograde transport of GDNF into the SNpc might play a less prominent role. This might confound the results for studies delivering GDNF only in the striatum of advanced PD patients, as employed by clinical trials conducted by Amgen (Lang et al., 2006; Nutt et al., 2003; Tatarewicz et al., 2007) and others (Slevin et al., 2005, 2007). Instead, other neurotrophic signaling molecules that could diffuse and elicit GDNF expression beyond the caudate putamen might extend the beneficial effects of GDNF into the SNpc.

Studies on the effects of PK2, specifically loss-of-function mutations, during neurodegeneration in wildtype adult mice are made difficult due to the developmental defects caused by a complete knockout of PK2. Therefore, a Cre-LoxP system which could conditionally and selectively ablate PK2 expression will be immensely useful in further studying the effects of PK2 compensatory responses, or a lack there of, during neurotoxic stress.

Other Future Directions: Role of PK2 in Neurogenesis

Preliminary data generated recently from our lab have demonstrated that PK2 participates in CNS neurogenesis in the subventricular zone (SVZ) lining the lateral ventricles. In the adult brain, the SVZ-derived neuroprogenitor cells migrate through the rostral migratory stream (RMS) in a chain of cells, eventually arriving at the olfactory bulb, where they tangentially migrate from the inner layers of the olfactory bulb to the outer layers. PK2 is upregulated by Ngn/MASH1 for olfactory bulb neurogenesis during development. The secreted PK2 most likely acts as a chemoattractant, as PKR2 can be detected on almost all migrating neuroprogenitor cells in the RMS (Ng et al., 2005). However, less is known about
PK2’s role in adult neurogenesis, and even less is known regarding its role in neurodegenerative processes such as in PD.

Environmental risk factors such as Mn exposure may lead to dysregulation of adult neurogenesis by reducing survival of neuroprogenitor cells as they migrate towards the olfactory bulb. The reduction of new olfactory bulb neurons affects not only the olfactory bulb functions, but also affect other brain regions such as the striatum, which is dependent on a continuous supply of neuroprogenitor cells to replenish lost neurons throughout adulthood and especially during neurodegeneration.

The role that PK2 plays in neurogenesis and determination of neuronal fate is incompletely understood. Through our *in silico* analysis, we have found the presence of a putative binding site for neuron-restrictive silencer factor (NRSF), that could suggest PK2’s involvement in key processes of commitment of neuronal cell fate or axon sprouting. NRSF represses expression of neuronal genes in nonneuronal cells and in NPCs by negatively regulating the activity of neuropilin-1, a positive regulator of axon branching promoted by the actions of VEGF and EGF, thus representing a possible negative regulator of PK2 during neurogenesis (Huang, Myers, & Dingledine, 1999; Jones & Meech, 1999; Kurschat, Bielenberg, Rossignol-Tallandier, Stahl, & Klagsbrun, 2006). These lines of evidence suggest that this balancing control aims to prevent the expression of PK2 and other co-regulated neurogenesis-associated genes such as neuropilin-1 until the precise moment that they are needed. In the same vein, vertebrate homologues of enhancer of split complex (HES) is another bHLH TF that was predicted to bind to the PK2 promoter. HES1 is critically important for sustainable neurogenesis (Dearden, 2015); decreasing expression of HES1 promotes the expression of proneural genes, while reducing the pool of neural stem cells. Interestingly, loss
of HES1 function leads to increased MASH1-positive NPCs in the olfactory placode, and results in excess neurogenesis (Cau, Gradwohl, Casarosa, Kageyama, & Guillemot, 2000). Thus, by antagonizing the activity of MASH1, HES1 works to provide a delicate balance between neurogenesis of new neurons and maintenance of neural stem cell pool. Further, the expression of PK2 is differentially upregulated as NPC differentiate into functional neurons while they migrate within the SVZ, and the upstream events that result in the cell’s decision to precisely upregulate PK2 at the most opportune moment remains unclear. Factors such as NRSF and HES1 involved in suppressing neuronal genes could provide such fine control of PK2 expression. The positive regulation of neural genes such as PK2 during neurogenesis by MASH1, countered by possible negative regulation by HES1 to repressing proneural identity, represent an exciting area of research. The perturbation of this delicate balance caused by exposure to Parkinsonian toxicants such as Mn deserves more attention.
Figure 1. Integrated summary of the role of PK2 signaling between neuron and astrocytes as presented in this dissertation. MPP⁺ damages could induce PK2 upregulation in neurons, which is secreted outside of the cell. In astrocytes, PK2, and PK2 receptor agonist IS20, could induce GDNF upregulation and secretion which could in turn stimulate cell survival in neurons.
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