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# Protein kinase C delta and prokineticin-2 signaling in experimental models of neuroinflammation and Parkinson's disease

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# Protein kinase C delta and prokineticin-2 signaling in experimental models of neuroinflammation and Parkinson's disease

by

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## ABSTRACT

Parkinson's disease (PD) is a progressive neurodegenerative disorder that was first described by the physician James Parkinson in 1817 as the shaking palsy. PD is now recognized as the most common neurodegenerative movement disorder affecting over 2% of the population and placing a severe economic burden on societies, particularly those with ageing populations. The pathological hallmark of PD is a progressive loss of dopaminergic neurons in the substantia nigra of the midbrain resulting in striatal dopamine depletion which manifests as a range of motor and nonmotor deficits. Progressive nigral dopaminergic degeneration is also accompanied by a chronic neuroinflammatory response that is characterized by increased microgliosis and the presence of dystrophic astrocytes. Research over the last decade has provided compelling evidence that the sustained microglial neuroinflammatory response can be neurotoxic and exacerbate dopaminergic degeneration. Therapeutic targeting of microglial activation has also been shown to mitigate dopaminergic degeneration in pre-clinical models of PD. However, the mechanisms by which microglial activation and neuroinflammation lead to dopaminergic neurotoxicity remain poorly understood. A major objective of this work was to characterize important signaling pathways activated during neuroinflammation, which could be therapeutically targeted to achieve neuroprotection in PD. Protein Kinase C delta (PKCδ), a novel member of the PKC family, has been shown to be an oxidative stress sensitive kinase that is proteolytically activated by caspase-3 following dopaminergic insults and mediates apoptosis of dopaminergic neurons. Significantly, blocking PKCδ using small molecule kinase inhibitors is neuroprotective in animal models of PD making it a potentially important therapeutic target for PD. However,

the role of PKCδ in regulating microglial activation and neurotoxicity has not been studied. Since the PKCδ signaling pathway is a crucial regulator of innate immune cell activation and proinflammatory responses outside the central nervous system (CNS), we hypothesized that PKCδ could be also be involved in regulating microglial activation and dopaminergic neurotoxicity during sustained neuroinflammation in PD. We tested this hypothesis using cell culture and animal models of neuroinflammation and PD. Herein, we demonstrate for the first time that PKCδ is proteolytically activated in dopaminergic neurons by tumor necrosis factor alpha (TNFα), an important proinflammatory cytokine, via the extrinsic cell death pathway to mediate dopaminergic degeneration. We also demonstrate that PKCδ is proteolytically activated in vivo using the intranigral LPS injection model of neuroinflammation and that targeting PKCδ protects against dopaminergic neurotoxicity induced by TNFα. In microglial cells, we show that PKCδ is highly induced during microglial activation and is a crucial regulator of microglial proinflammatory responses. Importantly, we demonstrate that microglia from PKCδ knockout mice have reduced proinflammatory responses and that PKCδ knockout mice are protected from dopaminergic degeneration in the MPTP model of PD. We also describe a novel method for isolation of pure primary microglia from postnatal mice by magnetic separation which provides the highest yield of microglial cells to date. While characterizing the effect of TNFα on dopaminergic degeneration, we serendipitously discovered that Prokineticin-2 (PK2), a novel member of the AVIT family, is highly induced in dopaminergic cells following neurotoxic insults. The final chapter of this work characterizes a novel and clinically relevant function for PK2 in the pathophysiology of PD and demonstrates for the first time that PK2 protects

dopaminergic cells from oxidative stress and neurotoxicity in vitro. These findings have immense implications for prokineticin-2 signaling in the pathophysiology of Parkinson's disease and establish a novel paradigm for prokineticin signaling during neurodegeneration and neuroinflammation. Collectively, the research described herein provides novel and important insight into PKCδ and Prokineticin-2 signaling during neuroinflammation and the pathophysiology of Parkinson's disease. These results have fundamental implications for therapeutic targeting of these crucial signaling pathways to mitigate progressive dopaminergic neuron loss in Parkinson's disease.

# CHAPTER I

# GENERAL INTRODUCTION

## Thesis Layout and Organization

The alternative format was chosen for this thesis and consists of manuscripts that are published, under peer review or being prepared for submission. The dissertation contains a general introduction, four research papers and a conclusion section that discusses the overall findings from all chapters. The references for each manuscript chapter are listed at the end of that specific section. References pertaining to the background and literature review as well as those used in general conclusion section are listed at the end of the dissertation. The introduction section under chapter I provides a background and overview of the idiopathic Parkinson's disease. The first background and literature review section covers current evidence implicating a pathogenic role for reactive microgliosis in mediating progressive dopaminergic neuron loss in Parkinson's disease. It also discusses the current knowledge of protein kinase C delta (PKCδ) signaling in dopaminergic degeneration and immune cell activation and provides an overview of the research objectives pertaining to chapters II, III and IV. The second literature review pertains to chapter V and provides an introduction to prokineticin-2 (PK2) signaling mechanisms and known physiological functions in the central nervous system and elsewhere. It covers the structural and functional aspects of PK2 signaling based on the current literature and provides contextual background for the research results described. The manuscript from Chapter II was recently communicated to the journal *Neurobiology of Disease* and Chapter III to the *Journal of Neuroscience*. Chapter IV describes a novel method for high-yield isolation of primary microglia by magnetic separation using Tetrametic Antibody Complexes (TACS) and has been published in the *Journal of Neuroscience Methods.* Chapter V describes research results pertaining to a novel

function for prokineticin-2 signaling during dopaminergic degeneration and is a manuscript that will be shortly communicated to the *Journal of Neuroscience.*

All of the research described by the author in this thesis was performed during the course of the author's doctoral studies at Iowa State University under the guidance of his major professor and principal investigator Dr. Anumantha G. Kanthasamy.

**Introduction**

Idiopathic Parkinson's disease (PD) is a chronic, progressive neurodegenerative disorder characterized by extrapyramidal motor deficits including akinesia, bradykinesia, rigidity and postural instability. First described by James Parkinson in 1817 as the shaking palsy, it is recognized today as the second most common neurodegenerative disorder affecting about 1 million people in the United States and over 4 million worldwide (von Bohlen und Halbach et al., 2004). Idiopathic PD has a median onset of around 60 years and its occurrence increases with age. The well-known pathological hallmark of PD is a significant loss of melanized dopaminergic neurons in the substantia nigra compacta, resulting in severe dopamine depletion in the caudate putamen. Typically, when motor symptoms are manifest at the time of diagnosis, around a 60% loss of dopaminergic cell bodies in the substantia nigra is evident with about an 80% depletion in striatal dopamine levels (Dauer and Przedborski, 2003). The conspicuous presence of Lewy bodies containing aggregated α-synuclein and other cellular degradation products is a characteristic feature of the disease in humans but is not reproduced well in animal models of PD. Reactive microgliosis is another prominent pathological feature of PD that is evident in clinical subjects and all animal models of the disease. Interestingly, although the extent of dopaminergic degeneration is greater in the striatum, reactive microgliosis is highest in the vicinity of dopaminergic cell bodies of the substantia nigra (Mosley et al., 2006; Bartels and

Leenders, 2007; Whitton, 2010). Until recently, the glial reaction accompanying nigral dopaminergic degeneration was presumed to be an innocuous consequence of extensive dopaminergic degeneration. Over the last two decades however, a wealth of evidence has accumulated that now strongly supports an active role for reactive microgliosis in the pathogenesis of PD, particularly in the progressive phase of the disease.

Various autonomic, non-motor symptoms have recently been associated with PD and are apparent in many but not all patients with the disease. The non-motor symptoms, which include sleep disorders, constipation, olfactory deficits and cardiovascular dysfunction, can in many cases precede the onset of motor symptoms by many years. Mild cognitive impairment is also seen in many PD patients (Magerkurth et al., 2005; Chaudhuri et al., 2006). Despite several decades of research into the etiology of PD, a cogent mechanistic basis for the selective vulnerability and progressive loss of nigral dopaminergic neurons has not been established. The emerging view appears to be that Parkinson's disease results from complex interactions between environmental, genetic and cellular processes that cause dopaminergic cell death over time, (Sulzer, 2007; Obeso et al., 2010b). A 'multiple-hit' hypothesis of disease onset has been proposed wherein different causative factors and multiple neurotoxic insults either accumulate over a lifetime or converge on the intrinsic vulnerability of nigrostriatal system, resulting in an idiopathic onset of PD (Carvey et al., 2006).

Six major genetic defects including alpha-synuclein, Parkin, DJ-1, LRRK2, PINK1 and Uch-L1 were identified as risk factors in certain genetic forms of PD but these account for fewer than 5% of the clinical cases, the rest being sporadic (Gwinn-Hardy, 2002; Dawson and Dawson, 2003; Vila and Przedborski, 2004). Environmental mechanisms, however, remain a major risk factor for development of sporadic PD. Several environmental factors including pesticides and heavy metals, well-water, pre- and post-natal exposure to

inflammatory agents and head trauma have been associated with an increased risk of developing PD (Di Monte et al., 2002; Lai et al., 2002). However, studies into the genetic forms of PD have provided fundamental insight into potential cellular and molecular mechanisms by which environmental factors can promote dopaminergic degeneration. Extensive research over the last few decades has shown that oxidative stress, mitochondrial dysfunction, and proteasomal impairment are important causative factors that can initiate dopaminergic degeneration in both idiopathic and genetic forms of PD (Przedborski, 2005; Jenner and Olanow, 2006; Olanow, 2007). However, neither oxidative stress nor mitochondrial and proteasomal dysfunction can by themselves sufficiently explain the progressive neuron loss that occurs in PD. Chronic neuroinflammation and sustained reactive microgliosis have been proposed to explain the progressive nature of dopaminergic degeneration in PD (Block et al., 2007a; Whitton, 2007). This hypothesis has recently gained increasing support from studies in experimental models of PD which demonstrate that microglial neurotoxicity can exacerbate local oxidative stress in the substantia nigra and sustain dopaminergic degeneration initiated by other idiopathic mechanisms (Block and Hong, 2005; Glass et al., 2010). Again, the precise mechanisms by which microglial cells become chronically activated and promote dopaminergic neuron loss remain to be established.

The emerging consensus suggests that PD is a complex, heterogeneous disease with multifactorial mechanisms of pathogenesis acting in concert with ageing to bring about disease onset and progression. The fact that many interacting factors appear to be responsible for the development of PD makes therapeutic intervention an immense challenge particularly at early stages of the disease (Obeso et al., 2010a). All of the approved treatment options for PD currently in use today including L-DOPA and deep brain stimulation only alleviate the symptoms of the disease and have no effect on the degenerative process. Therefore,

identifying common signaling pathways and therapeutic targets that are relevant across multiple modalities of disease pathogenesis would likely be an effective therapeutic strategy against PD. However, given the complex and heterogeneous nature disease pathogenesis, identifying such common downstream signaling pathways that are activated by distinct initiating mechanisms of disease pathogenesis would be difficult but necessary if early therapeutic intervention is to be achieved. Given that there are no known biomarkers for clinical PD that could enable early detection of dopaminergic degeneration, most patients are diagnosed at advanced stages of the disease process. Therefore, therapeutic intervention during the progressive phase of the disease process would be more feasible. Since neuroinflammatory processes and microglial neurotoxicity have been shown to be important mechanisms driving progressive dopaminergic neuron loss, targeting the microglial activation process and microglial neurotoxicity could be effective at slowing disease progression. Nonetheless, a more fundamental understanding of both the mechanisms of disease onset and its progression through neuroinflammatory processes would be necessary in order to rationally design effective therapeutic interventions.

## Background and Literature Review I

## Reactive Microgliosis and Protein Kinase Cδ Signaling Mechanisms in the Pathophysiology of Parkinson's disease

Neuroinflammation has been consistently documented in several chronic neurodegenerative diseases including Alzheimer's disease, Huntington's disease, Amyotrophic lateral sclerosis, and Parkinson's disease (Liu et al., 2003; Minghetti, 2005; Block et al., 2007b). Within the CNS, the hallmark of neuroinflammation is a persistent and localized activation of the resident brain glial cells, the astrocytes and microglia, at the site of neurodegeneration. For many years, the primary role of reactive gliosis accompanying neurodegeneration was intuitively believed to be phagocytic clearance and immune surveillance for activated microglia and neurotrophic support by astrocytes. During neuronal development and in the normal adult brain, activated glial cells participate in maintaining neuronal homeostasis during development and facilitate neuronal survival by providing neurotrophic support (Streit, 2002; Marin-Teva et al., 2004; Bessis et al., 2007). They also participate in neurogenesis and neural repair by directing the migration of neuronal progenitors to the sites of brain injury (Aarum et al., 2003; Walton et al., 2006). Rapid activation of microglia and astrocytes is also evident following acute brain injuries or infection and the gliosis that ensues is essential for neural repair and regeneration. In most cases of acute brain injury or infection, the neuroinflammatory responses are transient and subside once the infection is cleared or homeostasis is restored at the site of injury. In chronic neurodegenerative disorders such as Parkinson's disease, however, the neuroinflammatory

response is both chronic and progressive, with reactive gliosis being evident even in postmortem brains.

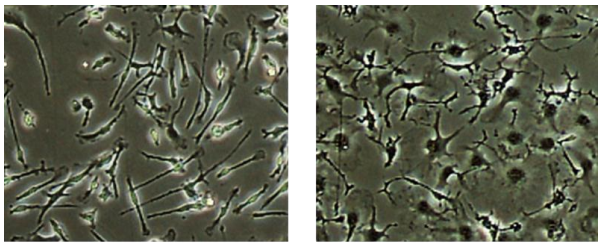
**Microglial activation and reactive microgliosis in Parkinson's disease**

Microglia are the resident macrophages in the brain and spinal cord. They were first described by Rio Hortega who identified their phagocytic properties and suggested that they could function similar to macrophages. This was confirmed several decades later in the 1988 by Hickey and Kimura when they demonstrated the perivascular microglia are antigen-presenting cells and express high levels of MHC class II. The exact origins of microglia were unclear until recently when it was confirmed that they originate in the bone marrow, are derived from myeloid cells and migrate into the brain early during development. Microglia make up about 10 to 15% of the total cells in the brain and their numbers vary according to region. The hippocampus, basal ganglia, olfactory telencephalon, and substantia nigra were found to have the highest microglial cell density in the CNS.

Microglial activation is the earliest and most evident response to neuronal injury in both acute and chronic neurodegenerative states (Figure 1). In the normal adult brain, microglia are typically present in their so called 'resting state' with a ramified morphology. In recent years, in vivo two photon microscopy has revealed that resting microglia are not dormant cells but are in fact highly dynamic and efficient in forming processes and scanning the neuronal microenvironment (Nimmerjahn et al., 2005). With adjacent microglia surveying overlapping regions of the brain parenchyma, recent studies estimate that the entire neuronal network of the brain can be scanned within a few hours (Davalos et al., 2005; Nimmerjahn et al., 2005; Hanisch and Kettenmann, 2007). In response to acute neuronal

injury or pathogenic stimuli, microglia undergo a rapid and dynamic transformation to reactive amoeboid forms with concurrent upregulation of various cell surface proteins including MHC molecules, cytokine and chemokine receptors. Again, in vivo imaging studies elegantly demonstrate that resting microglia can respond to chemoattractants and morphologically realign themselves within seconds or minutes. Transcriptional activation of several stimulus-specific sets of response genes also occurred rapidly in activated microglia with functional proteins being produced at around two hours. As would be necessary, the activation of microglia is both a dynamic and tightly regulated process relying on constant 'ON' and 'OFF' signals from neurons, astrocytes and the extracellular milieu and can be rapidly shut down once homeostasis has been attained (Kreutzberg, 1996)

**Figure 1: Morphology of resting and activated microglial cells**



Resting Microglia Activated Microglia

Activated microglia generate a large number of secreted factors including proteases, pro-inflammatory mediators, cytokines and chemokines that can alter the local neuronal microenvironment in the brain parenchyma in a stimulus-specific manner. Transient and regulated activation of microglia typically facilitate a microenvironment that promotes neuron survival and homeostasis (Glezer et al., 2007; Simard and Rivest, 2007; Napoli and Neumann, 2010). Activated microglia can respond with the release of anti-inflammatory and neurotrophic factors including BDNF, GDNF, IL-10 and TGFβ1 from microglia, which can stimulate anti-apoptotic survival signaling pathways including Akt/PI3K in neurons.

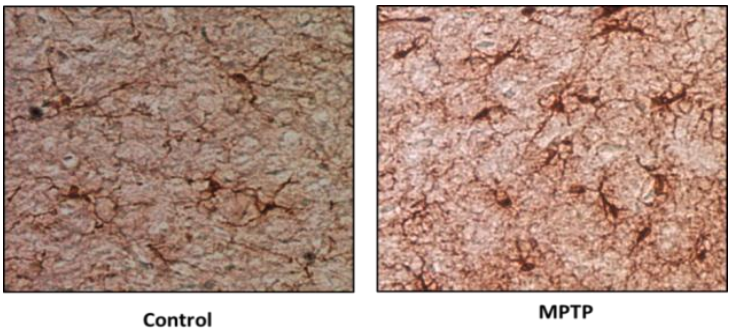
Microglia have been shown to protect neurons from excitotoxicity by upregulating GLT-1, a glutamate uptake protein under conditions where astrocyte glutamate uptake is impaired (Persson et al., 2005; Shaked et al., 2005). Microglial activation facilitates phagocytic removal of dead cells at the site of injury and can participate in regeneration of neural connections by synaptic stripping. In the entorhinal cortex, for example, activated microglia facilitate the physical removal of synapses and entire dendritic structures following neuronal injury. The synaptic stripping and phagocytic removal of cellular debris allow for the formation new synaptic connections and restoration of local neuronal homeostasis (Trapp et al., 2007). Microglia can directly promote neurogenesis and direct the migration of adult neural stem cells and in the brain. They can also negatively regulate neurogenesis by inducing a pro-inflammatory microenvironment (Butovsky et al., 2006)

**Microglial activation in neurodegenerative disorders**

In contrast to the transient and highly regulated activation profile seen during acute neuronal injury or during microglial housekeeping functions in the brain, microglial activation in progressive neurodegenerative disorders is morphologically and functionally distinct. Sustained microglial activation at the site of neuronal injury is a hallmark of most chronic neurodegenerative disorders. Prominent reactive microgliosis has been documented in postmortem brains of patients in most progressive neurodegenerative disorders. The microglia display their fully activated amoeboid state and increased microglial cell density at the sites of neurodegeneration. The microglial reaction observed in chronic neurodegenerative diseases has generally been regarded as an initial neuroprotective response. However, several lines of evidence from clinical and epidemiological studies,

animal models and in vitro now support the view that sustained neuroinflammation and reactive microgliosis can be neurotoxic and exacerbate progressive neuron loss. In the diseased brain microenvironment, persistently activated microglia can augment oxidative stress levels and secrete proinflammatory mediators, which can be neurotoxic over time (Aschner et al., 1999; Glass et al., 2010). The following section reviews the current evidence for microglial neurotoxicity in the pathophysiology of Parkinson's disease.

**Figure 2: Activated microglia cells in the substantia nigra of MPTP-treated mice**




**Experimental evidence for microglial neurotoxicity in the pathophysiology of Parkinson's disease**

**Clinical evidence and epidemiological data:** In postmortem studies of PD patients, extensive microglial activation is well documented in the nigrostriatal system, particularly around the most vulnerable melanin-containing dopaminergic neurons of the substantia nigra. Elevated levels of inflammatory mediators are also present in the cerebrospinal fluid of PD patients indicative of an ongoing inflammatory reaction that persists for the life of the patients (McGeer et al., 1988; Vawter et al., 1996; Banati et al., 1998; Whitton, 2007). Proinflammatory mediators, including TNFα, IL1β, and IFNγ, are markedly elevated in PD brains and the role in the degenerative process has been characterized in experimental animal models (McGeer et al., 1988; Hunot et al., 1997b; Mogi et al., 2000b; Hirsch et al., 2005).

The activation of caspase-3 and caspase-8 has been reported in PD patients with caspase activity being required for microglial activation (Hartmann et al., 2000; Hartmann et al., 2001; Burguillos et al., 2011). Microglial activation has been observed in the striatum and the rest of the basal ganglia by real-time PET imaging in living patients with idiopathic PD (Gerhard et al., 2006). Significantly, the microglial activation is evident in PD patients irrespective of the number of years with the disease when compared to healthy age-matched controls. Prominent nuclear translocation of NFkB, a crucial regulator of oxidative stress and proinflammatory responses, is evident in brains of PD patients (Hunot et al., 1997a). In support of the function of NFkB in PD, the use of highly specific NFkB inhibitors was shown to be efficacious in protecting against nigrostriatal dopaminergic degeneration in mouse models by mitigating the microglial neuroinflammatory responses and oxidative stress. Emerging evidence also suggests that the adaptive arm of the immune response might influence the degenerative process in PD. In postmortem patients, CD8+ and CD4+ T cells but not B cells were found in the substantia nigra in the absence of blood brain barrier damage (Brochard et al., 2009). Further, animal studies demonstrate that the T cells enter the brain following neuronal damage during the course of degeneration. Recent reports also suggest that the type of T cell response can both influence microglial activation and contribute to either dopaminergic neuron loss or neuroprotection (Benner et al., 2004; Reynolds et al., 2007; Kosloski et al., 2010; Reynolds et al., 2010). Postmortem analysis of humans who developed rapid-onset Parkinsonism after being exposed to the Parkinsonian toxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) show abundant reactive

microgliosis in the substantia nigra indicative of a sustained neuroinflammatory response that is perpetuated for the life of the disease (Langston et al., 1999).

Genetic linkage studies in cohorts of PD patients have also uncovered specific single nucleotide polymorphisms in promoter regions of proinflammatory genes that are linked to the development of PD or early onset PD possibly through transcriptional mechanisms (Kruger et al., 2000; Hakansson et al., 2005). The G-174C SNP in the IL-6 promoter is linked to the development of early onset PD in a study with 258 PD patients and 308 controls. Similar polymorphisms in position -511 of the IL-1beta gene promoter and position -1031 in the tumor necrosis factor (TNF) gene promoter have also been reported (Nishimura et al., 2000; Nishimura et al., 2001). The experimental evidence and confirmation by meta analyses for these promoter polymorphism studies, however, is lacking except in the case of TNFα which has been shown by independent research groups to have a neurotoxic role in PD pathogenesis in different animal models. Epidemiological studies provide further evidence implicating inflammatory mechanisms in PD (Schwarzschild et al., 2003). Continual use of non-steroidal anti-inflammatory drugs (NSAIDs) has been shown to lower the risk of PD by about 46% in human populations and this has been confirmed in animal models of PD (Chen et al., 2003; Chen et al., 2005a). More recent epidemiological studies, however, have shown that only certain classes of NSAIDs such as ibuprofen lower the risk for PD over a long period of time (Wahner et al., 2007). Strong epidemiological evidence also supports a role for caffeine consumption in decreasing the risk of developing PD (Checkoway et al., 2002).

**Evidence from neurotoxin-based models of Parkinson's disease:** Neurotoxin-based models of PD using rodents, non-human primates and cell culture systems have provided

valuable insight into disease pathology and molecular mechanisms since the early 1980 when MPTP was found to cause rapid-onset Parkinsonism in humans (Hisahara and Shimohama, 2010; Potashkin et al., 2010; Duty and Jenner, 2011). Although neither of the neurotoxin-based models recapitulates all of the pathological features of PD, they have been particularly useful in characterizing the microglial neuroinflammatory response, which correlates well between animal models and clinical pathology (Bartels and Leenders, 2007; Yokoyama et al., 2011). In fact, most of the findings from postmortem patients supporting a role for reactive microgliosis and neuroinflammation in PD pathology have also been verified using animal models of the disease. Reactive microgliosis and localized neuroinflammation in the nigrostriatal system are evident in all neurotoxin models of PD both in rodents and non-human primates (Czlonkowska et al., 1996; Kurkowska-Jastrzebska et al., 1999). Further, the magnitude of the microglial neuroinflammatory response is greater in the substantia nigra than in the striatum in animal models similar to what is seen in PD patients and postmortem human studies. Over the last decade, substantial evidence obtained with experimental animal models has strongly implicated a role for microglial neurotoxicity in the pathophysiology of PD, particularly in disease progression and delayed loss of dopaminergic neurons

The primary neurotoxin-based models of PD are the MPTP model and the 6-hydroxydopamine (6-OHDA) model, both of which recapitulate most of the cardinal symptoms of PD in non-human primates and rodents. The primary pathological mechanism of MPTP is the selective inhibition of complex-1 activity in dopaminergic neurons resulting in mitochondrial oxidative stress and delayed dopaminergic degeneration. A robust microglial neuroinflammatory response is evident in the substantia nigra of humans exposed

to MPTP and in non-human primate models of PD. In rodents treated with MPTP, microglial activation is accompanied by an increase in MHC class 1 and II expression and upregulation of iNOS in both the substantia nigra and striatum. Several pro-inflammatory cytokines and chemokines are also elevated with 24 hours of dopaminergic neuron insult including IL-1β, IL-6, IL-12 and TNFα (Grunblatt et al., 2001; Youdim et al., 2002). Fractalkine signaling was recently implicated in regulating microglial activation with dysregulated microglial activation and increased dopaminergic neurotoxicity evident in Fractalkine receptor (CX3CR1) knockout mice. Similar results were obtained with TNFα knockout mouse or TNF receptor knockouts, which showed better protection against MPTP-toxicity compared to wildtype mice. A similar neurotoxic role for FAS signaling and glucocorticoid receptor signaling involving increased microglial neurotoxicity has been demonstrated in using knockout mice with the MPTP model (Hayley et al., 2004; Ros-Bernal et al., 2011). The MAC-1/CD11b complex on microglia has also been implicated in microglia neurotoxicity using CD11b knockout mice that were shown to have reduced production of reactive oxygen species and dopaminergic neurotoxicity (Hu et al., 2008).

The most compelling mechanistic evidence for microglial neurotoxicity in the MPTP model comes from multiple studies and independent research groups, which have consistently demonstrated that microglial activation amplifies local ROS production in the substantia nigra to exacerbate dopaminergic degeneration. Components of the NADPH oxidase system are elevated following MPTP treatment in the mouse substantia nigra and also in human PD patients (Gao et al., 2003d; Wu et al., 2003; Zhang et al., 2004; Zhang et al., 2007d). The membrane-bound component of the NADPH oxidase is highly induced

following MPTP treatment and localizes to activated microglia, which were shown to be the primary producers of reactive oxygen species in vivo using dihydroedthidine histochemistry. Most importantly, NADPH deficient mice are protected dopaminergic degeneration and microglial oxidative stress in vivo (Wu et al., 2003; Qian et al., 2007). Targeting the NADPH oxidase system using small molecule inhibitor drugs or with antioxidants that scavenge the ROS species have been effective in protecting the nigrostriatal system from MPTP-induced damage with some being tested in clinical trials. Microglial iNOS is elevated following MPTP treatment and contributes to local ROS levels in the substantia nigra with knockout mice demonstrating robust neuroprotection from MPTP toxicity. (Kurkowska-Jastrzebska et al., 1999; Liberatore et al., 1999). Targeted inhibition of iNOS has also shown efficacy in the MPTP model of PD and in vitro. Signaling through the NFkB pathway regulates the production of several pro-inflammatory cytokines and chemokines as well as iNOS and components of the NADPH oxidase system in microglia and other innate immune cells. NFkB activation could therefore be a common downstream target of multiple activation pathways that result in downstream production of ROS species and pro-inflammatory mediators. Indeed, targeting NFkB activation using specific NBD peptide inhibitors effectively protects against dopaminergic degeneration in the MPTP model with attenuation of microglial neurotoxicity being a major protective mechanism (Ghosh et al., 2007; Ghosh et al., 2009).

**Lipopolysaccharide based neuroinflammatory animal models of PD:** Perhaps the strongest mechanistic basis and proof of concept for microglial neuoinflammation in the pathophysiology and progression of PD comes from animal and cell culture models using the

bacterial endotoxin lipopolysaccharide (LPS) to induce microglial neurotoxicity. LPS is a potent microglial activator and induces a strong inflammatory response and delayed dopaminergic degeneration when infused directly into the substantia nigra or given systemically (Castano et al., 1998b; Herrera et al., 2000; Castano et al., 2002; Hsieh et al., 2002; Qin et al., 2007). Importantly, the LPS-induced dopaminergic degeneration in mice appears to be permanent, being evident even up to a year after LPS injection. This suggests that transient inflammatory insults can initiate dopaminergic neurotoxicity which is sustained in a self-perpetuating manner (Liu et al., 2000; Castano et al., 2002; Gao et al., 2002; Block et al., 2007a). The LPS-based models are mechanistically distinct from neurotoxin models in that dopaminergic degeneration results from microglial neurotoxicity and not direct neuronal damage since LPS by itself is not directly toxic to neurons. Further, the results from neurodegeneration studies with LPS demonstrate that nigral neuroinflammation alone is sufficient to both initiate and sustain dopaminergic degeneration in the brain providing a rational for therapeutic targeting of the neuroinflammatory response.

In mice and rats, a single intranigral injection of LPS is sufficient to cause selective neurodegeneration of nigral dopaminergic neurons within two weeks, with the dopaminergic degeneration being evident even upto a year later. Nigral LPS infusion does not appear to damage to other types of neurons such as GABAergic and serotonergic neurons, or when injected in other regions of the brain, suggesting a selective vulnerability of the dopaminergic neurons to inflammatory insults (Castano et al., 1998a; Gao et al., 2002). Qin et al (2007) first demonstrated that a single intraperitoneal injection of LPS (5mg/kg) results in a protracted neuroinflammatory response in the brain long after the systemic inflammatory

responses in the serum and liver have subsided. Interestingly, the levels of TNFα remained in the brain even ten months after the systemic LPS insult while they returned to basal levels within a week in the serum and liver. In this model, dopaminergic degeneration becomes significant around ten months after the single LPS injection, although other modifications of this model with more multiple injections of systemic LPS can cause dopaminergic degeneration in two weeks (Qin et al., 2007; Lee et al., 2008). LPS can also act synergistically with MPTP and toxins including rotenone, paraquat and manganese to accelerate nigrostriatal degeneration (Gao et al., 2003a, c; Zhang et al., 2010b), implying that environmental toxicants, which have been heavily implicated as causative factors in idiopathic PD, can exacerbate neurotoxic inflammatory processes and vice-versa. In the presence of an LPS-induced neuroinflammatory response, the doses of neurotoxicants such as $MPP^+$ and rotenone required to cause dopaminergic neurotoxicity in mixed neuron-glial cultures are much lower than those required without the ongoing inflammation. Further, microglial ROS generation from the NADPH oxidase complex were shown to be required for this effect. Emerging evidence also suggests that prenatal exposure to LPS or other inflammogens in utero can prime the neuroinflammatory response in the adult mouse brain resulting in greater susceptibility to dopaminergic degeneration (Wang et al., 2009; Granholm et al., 2011). Further studies and epidemiological evidence would be required to conclusively demonstrate that exposure to inflammatory insults, either prenatally or during childhood, could be a risk factor for the development of PD several decades later in life.

The primary signaling mechanism relevant to LPS-induced microglial activation is NFkB nuclear translocation via TLR4 signaling with the subsequent activation of

downstream proinflammatory genes including iNOS, components of the NADPH complex and various cytokines and chemokines. (Qin et al., 2004; Wang et al., 2004; Dutta et al., 2008). The major neurotoxic mechanisms mediating dopaminergic degeneration in the LPS model are microglial ROS production via NADPH and iNOS as well as the production of proinflammatory cytokines, particularly TNFα (McCoy et al., 2006; Hunter et al., 2007; Qian et al., 2007; Cheret et al., 2008; Dutta et al., 2008). LPS-induced neurotoxicity on dopaminergic neurons can be effectively mitigated by various classes of drugs that target the microglial activation response including anti-inflammatory drugs, antioxidants, NADPH oxidase inhibitors, NFkB modulators and dominant-negative inhibitors of TNFα. In some cases almost complete preservation of the dopaminergic neurons in the substantia nigra has been demonstrated indicating potential for targeting neuroinflammation therapeutically in the progression of PD (Wang et al., 2004; Zhang et al., 2005a; McCoy et al., 2006; Qian et al., 2007; Hernandez-Romero et al., 2008; Zhang et al., 2010a).

**Signaling pathways regulating microglial activation during dopaminergic degeneration**

The mediators and signaling mechanisms responsible for sustained microglial activation in the substantia nigra during dopaminergic degeneration remain to be defined and are currently an active area of research. Although microglial activation through TLR-4 during LPS-induced dopaminergic degeneration is obvious, the signaling pathways regulating dopaminergic neuron-microglial interactions and the mechanisms regulating sustained activation of the microglia that eventually result in neurotoxicity are yet to be established.

**Table 1: Microglial activation factors relevant to dopaminergic degeneration**

| Activation Stimulus | Activation Mechanism | Functional Response in Microglia | References |
|---|---|---|---|
| **Alpha synuclein** | Phagocytosis from DA neurons | ROS generation<br>Cytokine production | (Zhang et al., 2005b; Lee et al., 2010) |
| **Neuromelanin** | Phagocytosis from DA neurons | ROS generation<br>NFkB activation<br>Cytokine production | (Wilms et al., 2003; Zhang et al., 2011) |
| **Active MMP-3** | Secreted from DA neurons<br>Possibly binds to protease receptors | ROS generation<br>Cytokine production | (Kim et al., 2005; Kim et al., 2007) |
| **HSP60** | Released from apoptotic DA neurons<br>Activates TLR4 | iNOS activation<br>Nitric oxide production<br>Microglial neurotoxicity | (Lehnardt et al., 2008; Stefano et al., 2009) |
| **RGS10** | Modulates Gα i/o signaling in microglia | RGS deficient microglia show increased activation &dopaminergic neurotoxicity | (Waugh et al., 2005; Lee et al., 2008) |
| **Fractalkine** | Negatively regulates microglial activation via fractalkine receptor signaling | Knockout mice show increased microglial activation & dopaminergic neurotoxicity | (Cardona et al., 2006; Shan et al., 2011) |
| **Gagnliosides** | Component of DA neuron membranes<br>Activate microglia by surface galectins | ROS generation<br>iNOS activation<br>cytokine production | (Pyo et al., 1999; Min et al., 2004) |
| **Paraquat &Rotenone** | Mechanism unknown | ROS generation<br>Potentiates microglial neurotoxicity | (Gao et al., 2003b; Wu et al., 2005) |
| **TNFα** | Signals through TNFR1 and TNFR2 | ROS generation<br>iNOS activation<br>nitric oxide production | (Barcia et al., 2011; McCoy et al., 2011) |
| **Diesel exhaust nanoparticles** | Phagocytosis by microglia | ROS generation | (Block et al., 2004) |

Recent studies have implicated both secreted proteins and intracellular components from degenerating dopaminergic neurons in the activation of microglia by diverse mechanisms and distinct functional outcomes. Secreted molecules from degenerating dopaminergic neurons include MMP-3, which can activate microglia through protease-activated receptors (Kim et al., 2007). Aggregated alpha synuclein and neuromelanin, which are abundant in degenerating dopaminergic neurons, have been shown to be taken up by microglial phagocytosis.(Wilms et al., 2003; Zhang et al., 2005b; Liu et al., 2007; Gao et al., 2008). Both aggregated alpha-synuclein and neuromelanin can promote NADPH oxidase activation and generation of ROS in primary microglia and drive dopaminergic degeneration in the substantia nigra and in mixed neuron-glial cultures (Wilms et al., 2007; Zecca et al., 2008; Zhang et al., 2011). Intriguingly, some of the signaling mediators released from injured dopaminergic neurons activate TLR-4 signaling similarly to LPS-induced activation of microglia. The known regulators of microglial activation during dopaminergic degeneration are summarized in Table 1.

**Mechanisms of microglial neurotoxicity during dopaminergic degeneration**

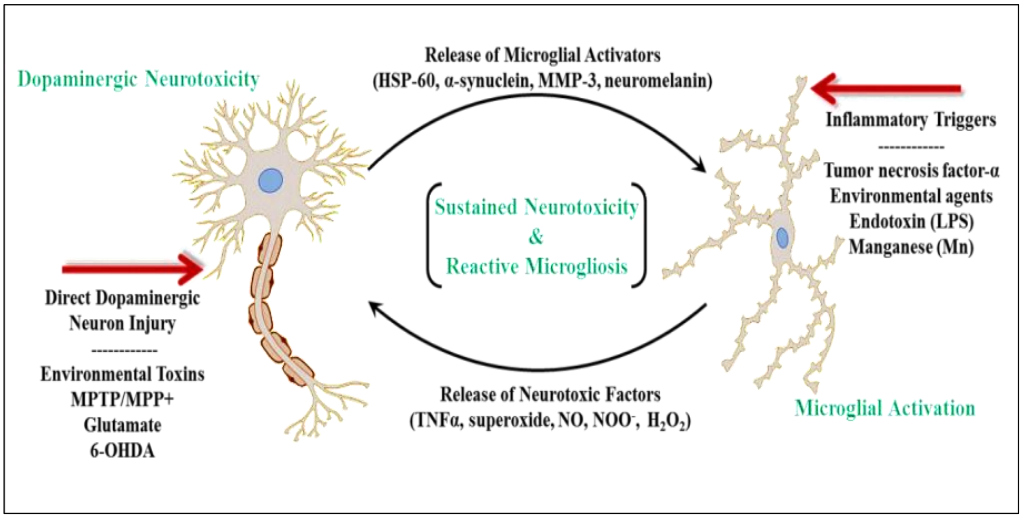
A wealth of evidence over the last decade from clinical studies, animal models and mechanistic in vitro experiments strongly supports a deleterious role for sustained microglial activation during progressive dopaminergic neuron loss (Whitton, 2007; Glass et al., 2010). Although it is now more widely accepted that persistent microglial activation can be neurotoxic, a mechanistic basis for microglial neurotoxicity on dopaminergic degeneration remains to be established. The substantia nigra is known to have a relatively higher population of resident microglia than most other regions in the brain (Lawson et al., 1990)

and this has been linked to increased dopaminergic neuron vulnerability to microglial neurotoxicity. For example, when LPS is injected at equivalent doses in other brain regions such as the hippocampus and cortex, no neurotoxicity is evident although robust microglial activation is apparent (Kim et al., 2000). Dopaminergic neurons also intrinsically exist in a pro-oxidant microenvironment due to their high dopamine and iron contents and their apparent glutathione deficiencies (Loeffler et al., 1994; Jenner and Olanow, 1996; Nakamura et al., 1997; Zeevalk et al., 1998; Zigmond et al., 2002) making them uniquely vulnerable to elevated levels of oxidative stress resulting from local microglial activation. As would be expected, the microglial activation response in the healthy brain is tightly controlled with inherent protective mechanisms to prevent excessive microglial activation that would be detrimental. Cannabinoids and glucocorticoids have been identified as potent endogenous signaling mechanisms that can negatively regulate the microglial inflammatory response and thus protect neurons from deleterious microglial activation (Nadeau and Rivest, 2003; McCarty, 2006; Rivest, 2006; Glezer et al., 2007; Cabral and Griffin-Thomas, 2008; Ros-Bernal et al., 2011). Similarly, the regulator of G-protein signaling 10 (RGS10) and signaling via the fractalkine receptor in microglia have both been identified as endogenous regulatory mechanisms that regulate microglial activation during dopaminergic degeneration. Both RGS-10 knockout mice and fracktalkine receptor knockouts show increased dopaminergic degeneration resulting from dysregulated activation of microglia (Lee et al., 2008).

The chronically activated, neurotoxic microglial response that accompanies dopaminergic degeneration implies that the inherently neurotrophic process of microglial activation gradually breaks down and is transformed into one that is deregulated and

progressively neurotoxic in the diseased state. Based on current evidence supporting a pathogenic role for sustained neuroinflammation and microglial neurotoxicity, it has been suggested that in PD, the degenerating dopaminergic neurons eventually lose their capacity to produce the signaling mediators that keep microglial activation in check, setting the stage for a vicious cycle of self-propelling neurotoxicity where uncontrolled reactive microgliosis further potentiates dopaminergic cell death (Figure 3). This line of thought fits well with the complex, multifactorial etiology that is thought to underlie the pathogenesis of sporadic PD. It also supports the multiple-hit hypothesis that has been proposed recently, wherein transient inflammatory and neurotoxic insults over the course of several decades can initiate a breakdown of regulatory mechanisms, resulting in a self-sustained process of chronic microglial activation and progressive dopaminergic neurodegeneration. Sustained microglial activation through ROS generation and proinflammatory cytokine responses can augment oxidative stress, which can further exacerbate the vulnerability of dopaminergic neurons.

**Figure 3: Self-sustaining mechanisms of reactive microgliosis and dopaminergic neurotoxicity in PD models**



**TNFα signaling during neuroinflammation and PD progression**

Recently, TNFα has emerged as an important signaling mediator that can act on both microglia and dopaminergic neurons to potentiate microglial activation as well as initiate direct dopaminergic neurotoxicity (Barnum and Tansey, 2011; McCoy et al., 2011). TNF signaling was first implicated in PD when both the soluble ligand and the TNFR1 receptor were found to be elevated in the brain and CSF of PD patients (Mogi et al., 1994; Bessler et al., 1999; Mogi et al., 1999; Nagatsu et al., 2000). In cohorts of PD patients, A specific TNFα promoter polymorphism (-1031 C), identified by two independent studies, appears to increase the risk of early onset of PD in cohorts of patients. (Nishimura et al., 2001; Wu et al., 2007). In these individuals, the polymorphism is believed to increase transcriptional activity at the promoter and increase TNF levels, potentially contributing to an earlier onset and progression of the disease.

TNFα expression is also increased in both the MPTP and 6-OHDA models of PD being elevated within hours of dopaminergic neuron insult in the substantia nigra. (Mogi et al., 1999, 2000a; Rousselet et al., 2002; Barcia et al., 2005; Ghosh et al., 2007). In the stereotaxic LPS models of nigral dopaminergic degeneration, a rapid increase in microglial TNFα production is one of the earliest activation responses (Herrera et al., 2000; Li et al., 2004). In primary microglial cultures, TNFα production is a common response to microglial activation by distinct stimuli relevant to dopaminergic degeneration (Table-1) including aggregated alpha-synuclein, gangliosides, activated MMP-3, neuromelanin and thrombin (Pyo et al., 1999; Wilms et al., 2003; Kim et al., 2005; Lee et al., 2005; Lee et al., 2010). In the systemic LPS injection model, TNF levels in the brain remain highly elevated for up to

10 months after the initial endotoxic shock, whereas the levels of TNFα in the serum and liver return to basal values within a few days (Qin et al., 2007). This suggests that the inflammatory response in the brain could be sustained for long periods of time after transient peripheral inflammatory insults and that TNFα may be play a key role in sustaining the inflammatory response in the brain.

Some of the strongest supporting evidence implicating a role for TNFα in mediating microglial toxicity and dopaminergic neuron loss comes from mechanistic studies using targeted inhibition of soluble TNF signaling with dominant negative mutants as well as experiments with knockout mice for TNF and its receptors. Mice deficient in both TNF receptors were shown to be resistant to MPTP-induced dopaminergic damage with attenuated microglial activation shown to be a potential mechanism involved (Sriram et al., 2002, 2006). However, in knockout mice lacking only a single TNF receptor (either TNFR1 or TNFR2), MPTP-induced striatal damage is reduced but no differences were seen in nigral neuron loss. Partial protection of the nigrostriatal system was also evident in TNFα knockout mice (Rousselet et al., 2002; Ferger et al., 2004). Dominant-negative mutants of TNFα were designed to inactivate signaling by soluble TNF but not by membrane bound TNF or reverse signaling by membrane bound forms (Steed et al., 2003). The dominant-negative mutants are engineered with specific mutations in the TNFα protein that almost completely inhibit binding and activation of TNF receptors and thus shutdown soluble TNF signaling. In both the LPS and the 6-OHDA model, dominant negative TNF effectively shuts down signaling by soluble TNFα and attenuates both microglial neurotoxicity and direct dopaminergic degeneration. In mixed neuron glial cultures, dominant negative TNFα also rescues loss of

TH-positive dopaminergic neurons when added to the cultures several hours after LPS or 6-OHDA treatments. (McCoy et al., 2008; Harms et al., 2011). These results were confirmed by independent studies in which dominant-negative TNF was stably expressed in the substantia nigra using adenoviral vectors. Again, the ablation of soluble TNFα signaling in vivo preserved the nigrostriatal system from 6-OHDA neurotoxicity and could rescue dopaminergic neuron loss (McCoy et al., 2008; Harms et al., 2011). On the other hand, the stable overexpression of endogenous TNFα using adenoviral vectors results in significant microgliosis and delayed dopaminergic neuron loss (De Lella Ezcurra et al., 2010).

Collectively, the evidence from PD patients, pharmacological studies and epidemiological data support a role for TNFα signaling as an important pathogenic mediator of dopaminergic neuron loss. The mechanism and signaling pathways, by which TNF elicits dopaminergic degeneration however are yet to be determined. Since receptors for TNFα are expressed on both dopaminergic neurons and microglia, it is possible that functional TNF signaling can occur in both these cells. TNF can potentiate microglial neurotoxicity and sustain microglial activation by maintaining high levels of ROS through NFkB activation (Block et al., 2007a; Gordon et al., 2011). Indeed, a recent study using TNF knockout mice demonstrated a critical role for TNFα and IFNγ in initiating and sustaining microglial activation in vivo (Barcia et al., 2011). Studies have also demonstrated that recombinant TNF can be directly toxic to dopaminergic neurons by mechanisms independent of microglial activation (Aloe and Fiore, 1997; McGuire et al., 2001; Carvey et al., 2005). Therefore, identifying key downstream signaling pathways activated by TNF in dopaminergic neurons

and microglial cells may provide additional therapeutic targets to mitigate dopaminergic neuron loss and further our understanding of TNFα signaling in dopaminergic degeneration.

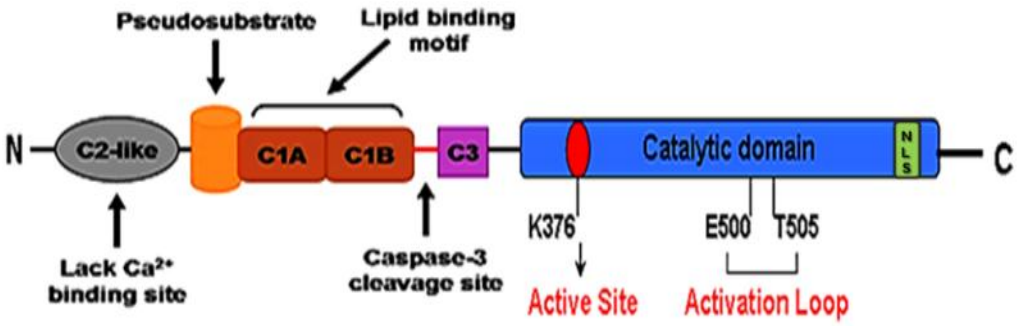
**Protein Kinase C delta signaling immune cells and experimental models of PD**

The protein kinase C (PKC) family is comprised of multiple related serine/threonine kinases that regulate diverse physiological processes including apoptosis and immune function. The PKC family consists of ten different isoforms classified into three distinct sub-family groups based on their functional activation requirements. Conventional PKCs include PKCα, $\beta_I$, $\beta_{II}$, and γ, which require intracellular calcium concentrations and diacylglycerol (DAG). The novel PKCs include PKCδ, ε, η, and θ. They are activated by DAG but are calcium independent. PKCζ and PKCλ make up the atypical PKCs, which require neither calcium nor DAG phospholipids for activation. The mature PKC isoforms are typically a single polypeptide with an N-terminal regulatory domain and a conserved catalytic domain containing the ATP binding site required for kinase activity. The regulatory domain at the N-terminus contains an autoinhibitory pseudosubstrate domain and two membrane-targeting domains (C1 and C2). The N-terminal regulatory domain is structurally and functionally distinct between the three sub-types of PKCs

**PKCδ protein structure:** Protein kinase C delta (PKCδ) was first cloned in 1987 from the rat brain. Being a member of the novel PKC subfamily, PKCδ contains a C-terminal catalytic domain with two conserved regions, C3 and C4 that are critical for catalytic competency and binding specific substrates. The amino terminal regulatory domain contains an auto-inhibitory pseudosubstrate sequence and two cysteine-rich Zn-finger-like sequences in the

C1domain that is essential for lipid binding. A caspase-3 cleavage site (SGN-**DIPD**-NNG) is present in the linker regions between the catalytic and regulatory domain. Proteolytic cleavage of PKCδ by caspase-3 is a common pro-apoptotic signaling mechanism in many cell types and constitutively activates the kinase. A nuclear localization sequence at the C-terminal catalytic domain between amino acids 610 and 630 regulates nuclear translocation of both full-length and proteolytically cleaved PKCδ. An activation loop is present on the catalytic domain of all PKC enzymes and is the site for priming phosphorylation events that are required for classical activation and translocation of PKCs to the membrane. In PKCδ the activation loop site is Threonine-505 (T505). A hydrophobic motif (S662) and a turn motif (S643) have been described for most PKC isoforms. Phosphorylation at specific serine residues in the hydrophobic motif also differentially regulates activation by various PKC agonists.

**Figure 4: Domain structure of PKCδ showing critical residues involved in activation and function**



**PKCδ activation mechanisms:** Like with most novel and conventional PKC isoforms, classical activation of PKCδ involves membrane translocation regulated by DAG binding to the cysteine-rich, zinc-finger-like motifs in the C1 domain. A series of sequential phosphorylation events at the C-terminus are required to prime the activation state and stabilize the PKC enzymes in a catalytically active, closed conformation that is resistant to protease degradation. Phosphorylation on the activation loop (Thr-505 in PKCδ) has been shown to align critical residues at the catalytic site but has been shown to be less important in PKCδ activation since the negative charge provided by the glutamate at position 500 (E500) can compensate for phosphorylation at T505. A glutamate-500 mutation (E500A) results in around 80% loss of PKCδ kinase activity. PKCδ has been shown to be catalytically active even in the absence of Thr-505 phosphorylation in vitro. Nevertheless, the kinase activity of PKCδ is increased considerably by phosphorylation at Thr-505. In PKCδ, the turn motif (Serine-643) has been shown to be an autophosphorylation site but its precise role, if any, in regulating PKCδ activity is unclear. Phosphorylation of PKCδ both at the turn motif and the hydrophobic motif appear to be stable in culture conditions and independent of most agonists unlike other PKC enzymes. Tyrosine phosphorylation has been identified as another crucial regulatory mechanism for PKCδ activation, translocation and substrate targeting. Key tyrosine phosphorylation sites (Tyrosine-52, -155, -187, -311, -332, -512, -523, and -565) have been defined in both the catalytic and regulatory domains of PKCδ. Phosphorylation at one or more of these tyrosine residues has been shown to regulate critical aspects of PKCδ activation in a cell and stimulus-specific manner (Steinberg, 2004). More recently, tyrosine

phosphorylation has also been shown to regulate translocation of PKCδ to the mitochondria and nucleus.

In addition to priming serine/threonine phosphorylation, membrane translocation and tyrosine phosphorylation, proteolytic cleavage of PKCδ by caspase-3 is a unique activation response that promotes apoptosis in various cell types including HeLa cells, salivary gland cells, keratinocytes, adenocarcinoma cells, and neutrophils (Ghayur et al., 1996; Pongracz et al., 1999; Reyland et al., 1999; Kanthasamy et al., 2003a). During apoptotic signaling, caspase-3 cleaves PKCδ adjacent to the Asp 327 at its recognition site (DIPD$^{\downarrow}$N) located within the hinge region between the catalytic and regulatory domains. Proteolytic cleavage releases the regulatory subunit from the catalytic subunit and yields a catalytically active 41-kDa fragment with constitutive kinase activity that is essential to promote apoptosis. Overexpression of caspase-3 cleavage-resistant mutants of PKCδ in various cells protects against apoptosis induced by diverse stimuli. Further, some studies have identified a potential positive feedback amplification loop between PKCδ and caspase-3 signaling.

**PKCδ signaling during dopaminergic degeneration:** PKCδ was first cloned from the rat brain and its expression in the developing and adult brain has been characterized in detail. PKCδ is expressed in different areas of the brain including the cerebellum, thalamus, habenula, and the septum (Barmack et al., 2000). PKCδ was also detected in glial cells and its expression varied ontogenically in both neurons and glial cells (Goldberg and Steinberg, 1996). Important functions for PKCδ have been described in the healthy brain and in various neurodegenerative disorders including stroke and AD. Previous studies from our laboratory have demonstrated that PKCδ is highly expressed in nigral dopaminergic neurons and can

bind to and phosphorylate tyrosine hydroxylase, the rate limiting enzyme in dopamine synthesis (Zhang et al., 2007b). Activation of caspase-3 has been shown to occur during dopaminergic degeneration in animal models of PD and active caspase-3 has been detected in postmortem PD brain. During dopaminergic degeneration induced by various neurotoxicants, caspase-3 activates PKCδ by proteolytic cleavage at the hinge region to promote neuronal cell death (Anantharam et al., 2002; Kanthasamy et al., 2003b; Kaul et al., 2003b). The proteolytic cleavage of PKCδ was shown to be dependent on phosphorylation at tyrosine-311 near the caspase-3 cleavage site (Kaul et al., 2005). Further, PKCδ can modulate upstream caspase-3activity via a positive feedback amplification loop resulting in sustained caspase-3 activation and amplification of apoptosis during dopaminergic degeneration (Kaul et al., 2003a; Kitazawa et al., 2003). Importantly, targeting PKCδ by pharmacological inhibition, siRNA knockdown or using PKCδ cleavage-resistant mutants effectively protects and rescues dopaminergic cell death induced by various neurotoxicants (Kaul et al., 2003b; Anantharam et al., 2004; Latchoumycandane et al., 2005; Kanthasamy et al., 2006; Sun et al., 2006). Further, therapeutic targeting of PKCδ using the small molecule kinase inhibitor rottlerin, could effectively protect against nigrostriatal degeneration in the MPTP animal model of PD demonstrating an important role for PKCδ during dopaminergic degeneration in vivo.

**Functions of PKCδ in immune cell activation and inflammatory responses:** PKCδ is ubiquitously expressed in immune cells and regulates various facets of the adaptive and innate immune response in addition to promoting apoptosis of immune cells. Some of the first studies using PKCδ knockout mice demonstrated an important role for PKCδ in

regulating B-cell tolerance. PKCδ knockout mice showed hyperactivation of B cells with autoimmunity and the presence of autoreactive antibodies to DNA and nuclear components (Mecklenbrauker et al., 2002; Miyamoto et al., 2002). In both B and T lymphocytes, nuclear localization of PKCδ mediates apoptosis in the absence of survival factors that prevent its nuclear accumulation of PKCδ (Scheel-Toellner et al., 1999; Mecklenbrauker et al., 2004). Similarly, the spontaneous apoptosis of neutrophils is mediated by PKCδ signaling (Kilpatrick et al., 2002).

A wealth of literature supports a role for classical PKCδ signaling in regulating the activation of innate immune cells and proinflammatory responses. Dendritic cell activation and antigen presentation was shown to be mediated by PKCδ signaling (Majewski et al., 2007; Guler et al., 2011). In human asthmatic eosinophils, PKCδ is required for migration, increased CD11b expression and MMP-9 granule release (Langlois et al., 2009). During macrophage activation by distinct stimuli, PKCδ participates in the production of TNFα, IL1β and IL10 (Meldrum et al., 1998; Lee et al., 2004; Leghmari et al., 2008). In acute pancreatitis, PKCδ was shown to mediate proinflammatory signaling by activating pancreatic NFkB, activator protein-1, and mitogen-activated protein kinases. Further, the targeted inhibition of PKC delta reduced myeloperoxidase activity and edema in the lung (Ramnath et al., 2010). Several reports have elucidated distinct functions for PKCδ in neutrophils with extensive crosstalk between TNFα and PKCδ signaling in these cells. PKCδ mediates the production of superoxide in TNFα-activated neutrophils (Kilpatrick et al., 2010) and directly regulates TNFα-dependent neutrophil survival signaling both at the level of the TNFR1 receptor and by modulating downstream survival signaling pathways (Kilpatrick et al., 2000;

Kilpatrick et al., 2002; Kilpatrick et al., 2006). PKCδ signaling also mediates the activation of NADPH oxidase and superoxide production in response formyl-methionyl-leucyl-phenylalanine (fmlp) in COS-7 cells (Cheng et al., 2007). Two recent studies also implicated a role for PKCδ signaling in mediating septic shock both systemically and in the lung with targeted inhibition of PKCδ effectively protecting against lung injury following sepsis.

**Summary and perspectives:**

Proapoptotic PKCδ signaling during dopaminergic cell has already been demonstrated using neurotoxins that activate caspase-3 via the intrinsic cell death pathway through mitochondrial oxidative stress. However, caspase-3 can also be activated via the extrinsic apoptotic pathway through death receptor signaling as in the case of TNF superfamily members. As described above, results from several studies support a neurotoxic role for TNFα in animal models of PD. Since classic TNFα death receptor signaling activates caspase-3, it is possible that TNF can promote the degeneration of dopaminergic neurons via the extrinsic apoptotic pathway involving downstream activation of PKCδ by caspase-3 mediated proteolytic cleavage. This hypothesis was explored in studies presented in chapter 2 of this dissertation which were designed to study the involvement of PKCδ in dopaminergic cell death by inflammatory mediators particularly TNFα. Given the known roles of PKCδ in immune function, particularly the activation of proinflammatory responses in innate macrophages and neutrophils, it is possible that similar signaling pathways be involved in the functional activation of microglial cells, which are brain macrophages that develop from the myeloid lineage. Evidence implicating PKCδ in microglial activation is limited. The PKCδ inhibitor rottlerin has been shown to inhibit LPS-induced microglial iNOS induction in N9

murine microglial cells (Byers et al., 1998). PKCδ was also shown to mediate paraquat-induced cell death in C6 glioma and BV-2 microglial cells (Miller et al., 2007). In studies with stroke models, microglial activation by thrombin was shown to induce tyrosine phosphorylation of PKCδ which could modulated the production of suppressor of cytokine signaling-3 (SOCS-3). Recently, caspase-3 activity was shown to regulate the microglial immune response and implicated PKCδ as a potential substrate for proteolytic cleavage in the process of microglial activation (Burguillos et al., 2011). Collectively, evidence indicates that PKCδ can potentially regulate microglial responses downstream of activation stimuli relevant to dopaminergic degeneration (Table-1). The studies performed in chapter 3 were based on this hypothesis and were designed to elucidate the potential functions of PKCδ in regulating microglial responses during activation by stimuli relevant to PD.

## Background and Literature Review II

### Prokineticin-2 Signaling

The Prokineticins (PK1 and PK2) constitute a pair of recently identified cysteine-rich secreted proteins belonging to the AVIT protein family that includes other homologs such as the mamba intestinal toxin-1 (MIT1) and endocrine gland derived vascular gland endothelial growth factor (EG-VEGF). The characteristic feature of the proteins in the AVIT family is the conserved N-terminal AVIT amino acid motif and the presence of 10 cysteine residues with almost identical spatial distribution that form a colipase-like fold motif with 5 disulfide bridges (Kaser et al., 2003; Bullock et al., 2004). The prokineticins were first identified and cloned in 2001 by three different groups and named based on their unique prokinetic property of gastrointestinal smooth muscle contraction. A decade of research since then has established that the prokineticins regulate a diverse range of physiological functions in different tissues including gut motility, circadian rhythms, angiogenesis, pain perception, hematopoiesis and olfactory bulb biogenesis. Recent evidence also suggests that prokineticins might be associated with pathologies of reproductive development, myocardial infarction, immune regulation and tissue-specific cancer initiation. The mature prokineticin protein forms of PK-1 and PK-2 are 86 and 81 amino acids long respectively. They have a predicted molecular weight of about 12 kDa and are highly conserved across species. PK-1 and PK-2 have around 45% identity in their amino acid sequences and are expressed on different chromosomes. In humans, the PK1 gene is located on chromosome 1p21 while PK2 maps to 3p21.1. A remarkable aspect of prokineticin signaling is the ability of these two ligands to regulate such complex and diverse biological processes. The emerging consensus

suggests that the differential expression of the two prokineticins and their receptors by tissue-specific gene regulation networks might account for this diversity of functions.

**Figure 5: Amino acid sequences of PK1 and PK2 depicting the conserved AVIT motif and ten cysteine residues**


**Prokineticin Receptors**

A few years after the initial discovery of prokineticins, their two G-protein coupled receptors PKR1 (GPR73A) and PKR2 (GPR73B) were identified and cloned (Lin et al., 2002a; Masuda et al., 2002). The two prokineticin receptors belong to the neuropeptide Y family and have an unusually high degree of similarity (~85% amino acid identity) with most of the variation around the N-terminal region. Human PKR1 is located on chromosome 2q14 and PKR2 on 20p13. The two GPCRs couple to Gq, Gi or Gs in a cell-specific manner to mobilize intracellular calcium and turnover of phosphoinositol and cyclic AMP accumulation. The specific signaling pathways activated downstream of the prokineticin receptors however remain to be elucidated. Interestingly, both prokineticin ligands PK1 and PK-2 can bind to either receptor with apparent non-selectivity (Lin et al., 2002a) suggesting that the expression and availability of the ligand and receptor pair are likely to determine the specific signaling axis that is involved in a tissue-specific physiological process. Both

prokineticin receptors have been shown to be expressed in the ovaries, testes, adrenal glands, uterus and intestinal tract. In the bone marrow and the blood, both PKR1 and PKR2 have been detected in mature blood cells and hematopoietic stem cells (Lin et al., 2002a; Soga et al., 2002; LeCouter et al., 2003; Battersby et al., 2004; Hoffmann et al., 2006, 2007). In the placenta and the heart, PKR1 appears to be the major prokineticin receptor that is involved in regulating cardiomyocyte survival. (Urayama et al., 2007). In the brain, PKR2 is more ubiquitously expressed than PKR1 and predominantly in the regions associated with olfactory bulb biogenesis and circadian regulation where the role of PK2-PKR2 signaling has been functionally characterized (Cheng et al., 2006).

Studies using transgenic overexpression of the prokineticin receptors and knockout mice have provided much insight into the role of each prokineticin receptor in distinct physiological processes. Loss of function mutations in either PK2 or PKR2 have been shown to result in human Kallmann syndrome and hypogonadotropic hypogonadism with the primary pathological features being recapitulated in mouse models with either receptor knockouts or null mutations (Pitteloud et al., 2007; Abreu et al., 2008). PKR2 but not PKR1 knockout mice show abnormal olfactory bulb development with hypoplasia and severe atrophy of the reproductive organs highlighting the importance of PKR2 signaling in both these physiological processes. PKR1 knockout mice on the other hand have impaired pain sensitization and nociceptive responses that are also evident in PK2 knockout mice indicating the involvement of a PK2-PKR1 signaling pathway in these events (Hu et al., 2006; Negri et al., 2006; Giannini et al., 2009). In the cardiovascular system where both receptors have distinct spatial expression patterns, overexpression of PKR2 in mouse hearts induces

hypertrophy and capillary vessel leakage without angiogenesis whereas the targeted overexpression of prokineticin receptor-1 (PKR1) induced neovascularization implicating different distinct signaling pathways for the two receptors in this system (Urayama et al., 2008; Urayama et al., 2009). In contrast, PKR1 knockout results in dysfunctional cardiac and kidney development and is accompanied by mitochondrial defects and increased apoptosis in both tissues (Boulberdaa et al., 2011). The results from studies with the individual prokineticin receptor knockout mice further underscore the fact that differential expression and availability of the individual prokineticin ligands and receptors could account for their ability to regulate the above set of complex and diverse biological processes.

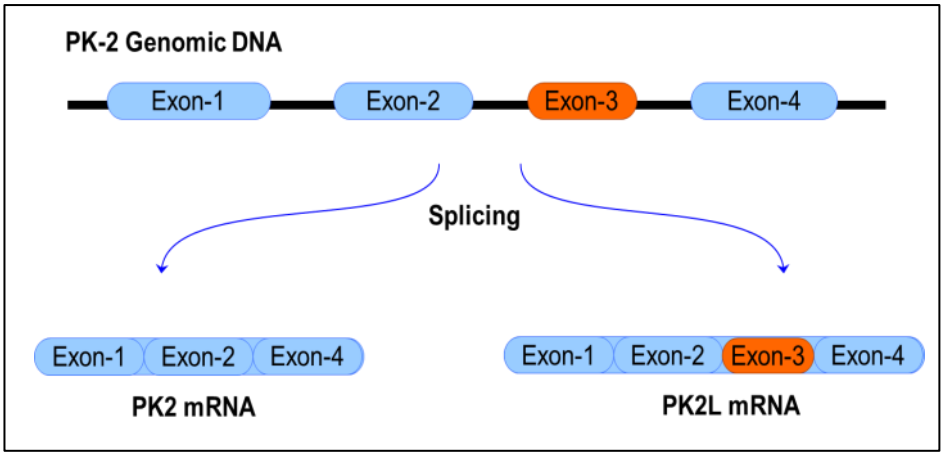
Given that the prokineticins and their receptors were discovered only around a decade ago, much remains to be understood in terms of tissue-specific transcriptional regulatory networks that define the expression patterns of each receptor as well as the downstream signaling pathways coupled to them. More recent studies are shedding light on some of these paradigms. For example, emerging evidence suggests that PKR2 potentially exists as a dimer in vivo with interactions between transmembrane domains being important for dimerization. (Marsango et al., 2010). A recent study also identified connective tissue growth factor (CTGF) as a direct downstream target of a PK1-PKR1 signaling in endometrial epithelial cells and decidua samples.

**Prokineticin-2 gene structure**

Prokineticin proteins are assembled from relatively simple mRNA elements made up of the signal peptide followed by the mature functional protein. The gene and promoter structures are highly conserved in both humans and mice, indicative of their functional roles

in important physiological processes. Mammalian prokineticin genes contain four exons with the third exon being a target for alternative splicing in prokineticin-2. The first exon encodes a 19-residue signal peptide that targets the proteins for constitutive secretion. Exon-2 contains six of the cysteine residues and Exon-4 encodes for four of the ten conserved cysteines. An alternative splice form of prokineticin-2 has been identified recently, which contains a 63 bp insertion between exon 2 and exon 4 resulting in a longer isoform of PK-2 (PK2L) that has an additional 21 amino-acid residues (Kaser et al., 2003; Chen et al., 2005b).

**Figure 6: PK2 gene structure and alternate splicing forms**



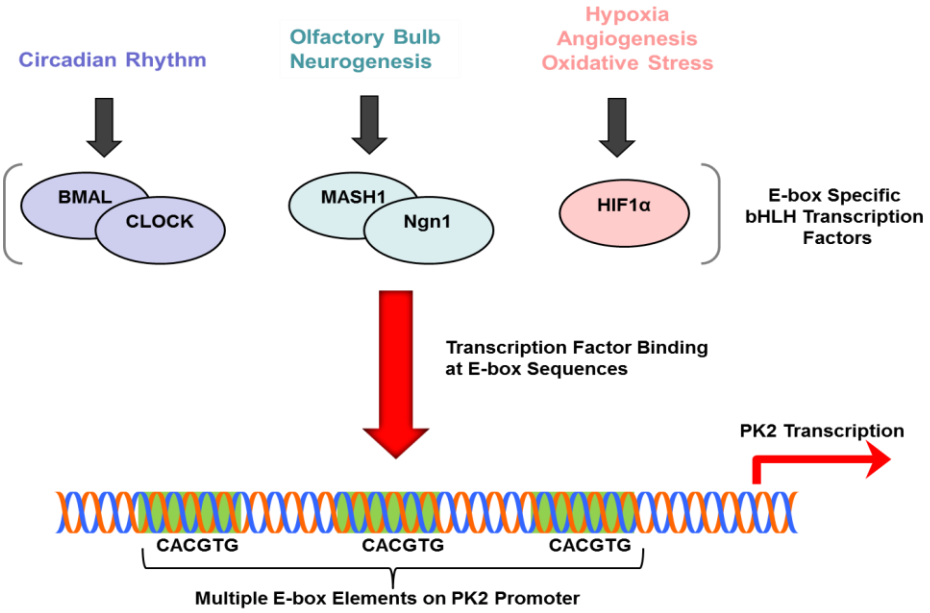

In the mature PK2L isoform, the 21 amino acid insertion occurs between Lys47 and Val48 and consists of highly basic amino acids with 19 being either lysine or arginine. Further, exon-3 insertion encodes for two putative furin protease cleavage sites. Co-expression of furin with PK-2L in vitro resulted in a shorter cleaved form of the protein (PK2β) that could still functionally activate prokineticin receptors (Chen et al., 2005b). Interestingly, the shorter PK2β fragment showed greater selectivity for PKR1 than PKR2 whereas both PK1 and PK2 show no selectivity for either prokineticin receptor. The specific

function of the alternatively spliced isoform (PK2L) is yet to be determined in vivo although preliminary evidence suggests it might be differentially expressed from PK2 in some tissues such as the lung (Chen et al., 2005b). No alternative splice forms have been detected for PK1 to date. Prokineticins are highly basic proteins with the pI of PK-2 determined to be around 8.85 while that of PK2L was 10.68 due to the lysine and arginine rich exon-3 insertion. PK1 (EG-VEGF) was shown to bind with high affinity to heparin-sepharose, and it has been suggested that due to the highly basic nature of these secreted proteins, their bioavailability and activity may be further regulated by interactions with components of the extracellular matrix (LeCouter et al., 2003).

The transcriptional regulatory elements for PK2 have been characterized in detail for with the human and mouse promoters being highly conserved between the two species (LeCouter et al., 2003; Zhang et al., 2007a). Multiple E-box elements (CACGTG) have been identified in both the mouse and human PK-2 promoter sequences (Cheng et al., 2002; Zhang et al., 2007a). The E-box sequences are targets of a diverse set of transcription factors belonging to the E-box specific basic Helix-Loop-Helix (bHLH) family, which include CLOCK, BMAL1, Ngn1, MASH1 and HIF1α, all of which have been shown to regulate the expression of PK2 in distinct physiological processes. The expression in the suprachiasmatic nucleus was shown to be under the control of CLOCK and BMAL (Cheng et al., 2002; Cheng et al., 2005; Watanabe et al., 2007). During olfactory bulb biogenesis where PK2 is abundant in the granule cell layer and periglomerular layers, its expression is regulated by the proneural transcription factors Neurogenin 1(Ngn1) and MASH1 which directly bind to E-box elements in the PK-2 promoter. These results were also confirmed in vivo with Ngn1 and

MASH1 mutant mice which showed significantly reduced PK-2 expression(Zhang et al., 2007a). Two putative HIF1α binding sites were defined on the mouse promoter less than 300 nucleotides upstream of the transcriptional start site at -115 and -248. Indeed, PK2 was shown to be highly induced by hypoxia both in vivo and in cell cultures (LeCouter et al., 2003; Shojaei et al., 2007). Thus, the ability of PK-2 to regulate many distinct biological processes ranging from olfactory bulb development to angiogenesis and circadian function can be partly attributed to its sophisticated mechanisms of gene regulation by different members of the bHLH family of transcription factors.

**Figure 7: Transcriptional regulation of PK2 in distinct biological processes by bHLH transcription factors**

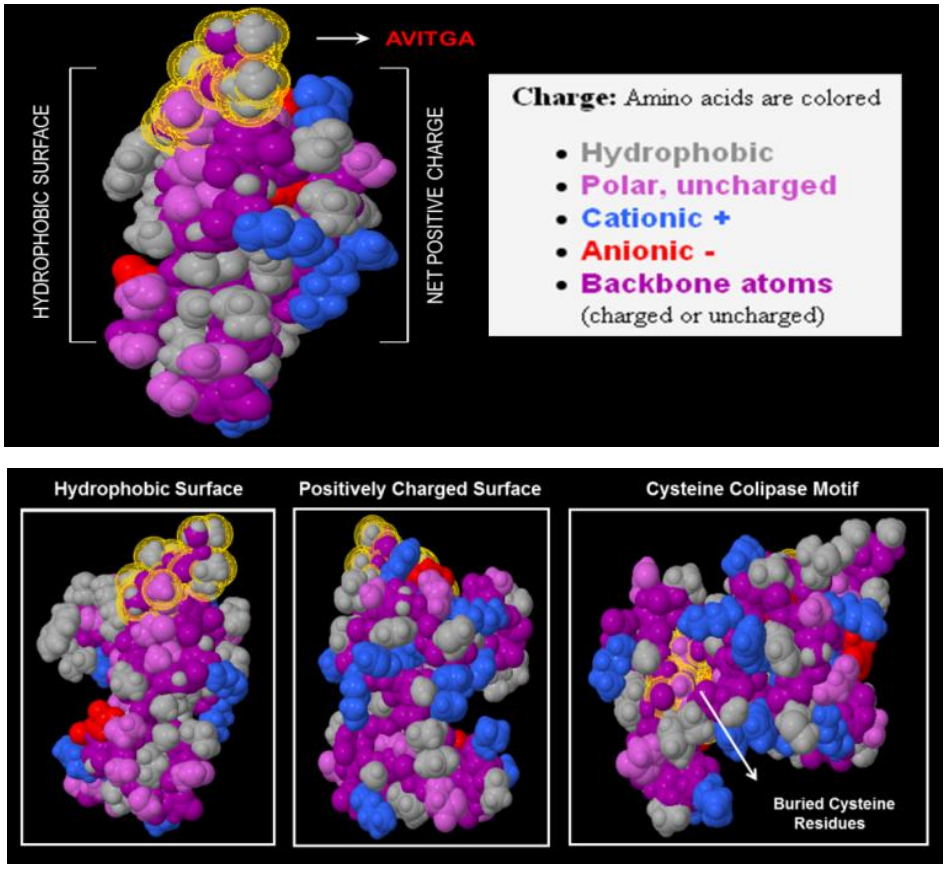



**Prokineticin-2 protein structure**

Current insight into the structural elements of prokineticin-2 is largely based on the known NMR structure of MIT-1, the prototypical member of the AVIT family and the recently

published NMR structure of the amphibian prokineticin homolog Bv8 (Boisbouvier et al., 1998). The prokineticins have a very compact structure as a result of the five disulfide bridges formed between the 10 highly conserved cysteine residues buried in the core of the folded protein. The N-terminal and C-terminal ends are exposed at the surface of the molecule and participate in receptor binding and activation. The folded protein is believed to have an approximately ellipsoid shape with a net positive charge on one side and a hydrophobic face on the other (Fig. 8).

**Figure 8: PK2 protein structure depicting spatial distribution of charged amino acids and buried cysteine residues in the folded protein**



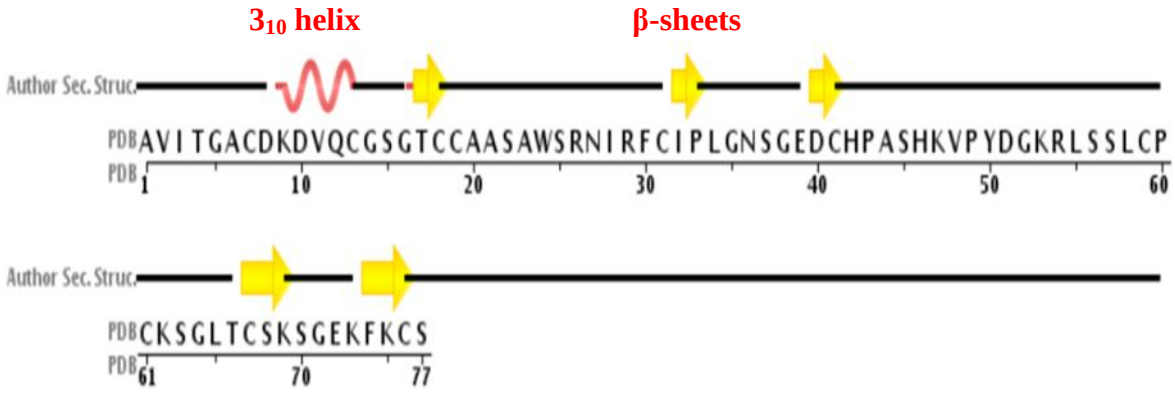
Many charged amino acid residues are buried inside the core of the protein in proximity with the disulfide bridges whereas some of the hydrophobic residues are more exposed on the surface. The more recent and detailed NMR structure of the amphibian prokineticin homolog also identified two β-hairpins at the C and N terminus of the protein and a single turn of $3_{10}$ helix at the N terminus (Kaser et al., 2003; Morales et al., 2010).

The structural and functional elements of the protein required for signaling have also been characterized in detail using point mutation analysis and by generating chimeric prokineticins. The first six N-terminal amino acids (AVITGA) of both prokineticins and all of their invertebrate homologs are completely conserved and are critical for both receptor binding and activation. Addition or substitution mutations in any of the six amino acids results in an inactive protein. However, the N-terminal AVITG segment alone appears to be insufficient for activity as synthetic peptides of the first 6 or 12 amino acid residues alone does not have any significant functional activity even at high doses (Bullock et al., 2004). NMR studies also revealed that deletion of the AVITG region did not alter the overall structure and fold of the Bv8 homologue but results in a complete loss of activity. Further, the loss of activity in the N-terminal truncated AVIT-mutant could not be compensated for or complemented by the addition of synthetic AVITG-OH or AVITG-$NH_2$ peptides in solution. Interestingly, the mutant lacking the AVITG motif also failed to antagonize the functional activity of the native protein suggesting that the N-terminus of the protein is probably required for both receptor binding and receptor activation. (Morales et al., 2010). The contribution of the C-terminal amino acids and the cysteine residues have also been investigated by mutation analysis and domain swapping experiments. Substitution of two of

the cysteine residues (cysteines 18 and 60) abolished prokineticin signaling in functional assays. Importantly, substitution of the cysteine rich C-terminal domains from other protein families such as the colipase and dickkopf proteins failed to elicit activation of the prokineticin receptors. However, the swapping of C-terminal domains of PK1 and PK2 with the C-terminal domains of each other preserved their functional activity (Bullock et al., 2004). Thus, while the specific framework of the cysteine residues is critical to functional activity, there appears to be some tolerance to variability in the C-terminus based on the fact that PK-1 and PK-2 which are only about 50% identical in their amino acid sequences can still functionally substitute for each other at the C-terminal domains.

Together, the data from genetic, biochemical and structural studies to date suggest that the binding of the prokineticins and activation of their receptors proceeds through a highly intricate process. The N-terminal AVITG motif is absolutely necessary for binding and functional activation of the prokineticin receptors. Similarly, the spatial distribution of the conserved cysteine residues at the core of the protein appear to be critical for activity while a small degree of tolerance to variations in the C-terminal domain might be plausible. The availability of the recently solved NMR solution structure of the Bv8 would facilitate a better understanding of the structure-activity relationships regulating the dynamics of prokineticin ligand-receptor interactions. For example, identifying the role of the conserved amino acid residues situated in the solvent-exposed loops of the protein would likely provide further insight into cooperative interactions involved in prokineticin ligand-receptor binding and functional activation (Morales et al., 2010)

**Figure 8: PDB secondary structure for PK2 based on the NMR structure solved by Morales et al (2010).**




An important aspect of understanding the structure-function relationships involved in prokineticin signaling would be the development of agonists and antagonists to the G-protein coupled receptors PKR1 and PKR2. Elegant studies by Bullock et al. (2004) using prokineticins with N-terminal substitution mutants identified that specific mutations at the N-terminus can result in mutant proteins with antagonist activity. Substitution of the N-terminal alanine with methionine (A1MPK1) or the addition of a methionine at the N-terminus (MetPK1) generates mutant proteins that antagonize the activity of PKR1 and PKR2 by both PK1 and PK-2. Further, the pre-incubation of the antagonists increased their potency by almost twofold. The peptide antagonists were also shown to be functional in vivo and in cell culture assays and have been useful in dissecting the functional roles of prokineticin receptor signaling in the brain(Cheng et al., 2002; Cottrell et al., 2004; Ng et al., 2005). Chemical antagonists of the prokineticin receptors have been recently developed based on triazine and pyrimidindione derivatives (Balboni et al., 2008; Giannini et al., 2009; Ralbovsky et al.,

2009). The triazine-based compounds were found to be highly selective for PKR1 in functional assays with almost 70-fold less potency as a PKR2 antagonist. Newer generation of traizine antagonists have been tested in models of inflammatory pain and have been found to be highly effective as anti-hyperalgesic drugs (Giannini et al., 2009)

**Downstream signaling pathways activated by prokineticins:**

The two prokineticin receptors belong to the neuropeptide Y (NPY) receptor class of GPCRs which promote intracellular calcium mobilization, cAMP accumulation and phosphoinositol turnover (Lin et al., 2002a; Masuda et al., 2002). The first study to clone and characterize the prokineticins demonstrated that the recombinant proteins caused calcium influx at nanomolar doses and that the calcium influx was essential for their prokinetic actions on gastrointestinal smooth muscle. Preliminary evidence that prokineticins signaled through GPCRs came from GTPγS displacement experiments by the same study (Li et al., 2001). Since then, various groups have shown that the two prokineticin GPCRs couple to Gi, Gq and Gs proteins depending on the cell type to activate multiple signaling pathways (Chen et al., 2005b). In adrenal cortex-derived endothelial (ACE), PK1 (EG-VEGF) activates p44/42 MAPK in a dose- and time-dependent manner which could be blocked by pertussis toxin, implicating $G\alpha_i$ coupling. Activation of the MAPK pathway resulted in increased proliferation and migration of these cells in response to PK1. Further, PK1 treatment also activated Akt and endothelial endothelial nitric-oxide synthase (eNOS) in an Akt-dependent manner. Akt activation was also a crucial survival signal for these cells in response to prokineticin signaling (Lin et al., 2002b). In endometrial epithelial Ishikawa cells, PROK1 induced the expression and secretion of IL-8 and IL-11 via the calcineurin/NFAT signaling

pathway (Maldonado-Perez et al., 2009; Cook et al., 2010). The expression of connective tissue growth factor was induced by PK1 in these same cells and was dependent on Gq, phospholipase C (PLC), cSrc, epidermal growth factor receptor (EGFR), mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling pathways based on inhibitor studies (Waddell et al., 2011). In longitudinal muscle-myenteric plexus cultures, treatment with PK1 stimulated nitric oxide release, a potential mechanism by which PK1 inhibits spontaneous giant contractions in the circular muscle of the mouse proximal colon (Hoogerwerf, 2006). PK1 also activates the Akt pathway and induces proliferation and survival of neuroblastoma cells. Interestingly, PK1-induced proliferation required the expression of both prokineticin receptors while PKR2 was essential in mediating cell survival. In enteric neural crest cells, similar signaling pathways including the MAPK and Akt signaling are both activated by PK1 to induce proliferation and differentiation (Ngan et al., 2007a; Ngan et al., 2007b). Similar survival signaling pathways are activated by in multiple myeloma cells where PK1 caused the phosphorylation of MAPK, Akt and STAT3. PK1 also upregulated Sphingosine kinase (SphK) and the potent anti-apoptotic bcl-2 family member Mcl-1 in these cells demonstrating that multiple survival signaling pathways could be activated downstream of the prokineticin receptors (Li et al., 2010). PK1 also has proliferative effects on bovine steroidogenic glomerulosa and fasciculata cells in a pertussis toxin-sensitive manner indicating $G_i$ coupling to the prokineticin receptors in these cells. PK1 treatment resulted in phosphorylation and activation of ERK1/2, which could be responsible for its proliferative effect in these endocrine cells, where prokineticins are thought to act as autocrine mitogenic factors (Keramidas et al., 2008). In the third trimester

placenta, PK1 is highly expressed and was defined as a novel mediator of the inflammatory response since it induced the expression of IL-8 and COX-2(Denison et al., 2008). In placental cell extracts PK1 activated ERK1/2, which was dependent on sequential phosphorylation of cSrc and the epidermal growth factor receptor (EGFR). In both placental endothelial cell (HPEC) and umbilical vein-derived macrovascular endothelial cells (HUVEC), PK1 also acts as a survival factor by activating Akt and MAPK signaling (Brouillet et al., 2010). In human peripheral blood monocytes, PK1 induces morphological changes and the down regulation of B7-1, CD-14, CC chemokine receptor 5 and CXC chemokine receptor 4 (Dorsch et al., 2005). Further, monocytes treated with PK1 had increased levels of IL-12 and TNFα and decreased IL-10 production in response to LPS stimulation. Other groups showed that monocytes but not macrophages or dendritic cells stimulated with PK1 produced the chemokines CCL4, CXCL1 and CXCL8 (Monnier et al., 2008).

Since both PK1 and PK2 activate each of the prokineticin receptors (PKR1 and PKR2) with similar potency and show no selectivity to either receptor, their downstream signal transduction pathways are likely to be similar. PK2 acts as a potent chemotactic factor for macrophages at doses in the subnanomolar range. PK2 also potentiated LPS-induced IL-1 and IL-12 production and reduced IL-10 levels in these cells (Martucci et al., 2006). The effects of PK2 on macrophages were found to be pertussis toxin insensitive but were blocked instead by the phospholipase inhibitor U73122 indicating the involvement of Gq proteins downstream of the prokineticin receptors. Interestingly, studies with prokineticin receptor knockout mice demonstrate that all of the effects of PK2 on macrophages are mediated by

PKR1. In primary mouse splenocytes, a similar pathway involving PK2-PKR1 signaling was demonstrated in vitro and in vivo using receptor knockout mice. PK2 increased the production of IL-1beta and decreased IL-4 and IL-10 levels in cultured mouse splenocytes and these effects were abrogated in cultures from PKR1 knockout mice. PK2 was also demonstrated to be a potent chemotactic factor for neutrophils most likely via PKR2 signal transduction since PKR1 was not detected in these cells. PK2 was highly expressed in neutrophils that infiltrated tumors and isolated PK2 from neutrophils was found to be fully active in vitro. Further, the expression of PK2 in isolated neutrophils was induced by granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) in these cells. All of the reported effects of PK1 and PK2 on hematopoiesis and mobilization of hematopoietic cells were found to be pertussis toxin sensitive implicating the involvement of $G_i$ proteins in prokineticin GPCR signal transduction in haematopoietic cells. In cardiomyocytes, a PK2-PKR1 signaling pathway was elegantly described using receptor-specific siRNA. PK2 activated Akt in these cells to protect against oxidative stress and promote angiogenesis (Urayama et al., 2007).

In neuronal cells and brain slices, PK2 can influence cell excitability presumably via PKR2, which is the dominant prokineticin receptor expressed in the brain. Following the initial discovery that PK2 regulates circadian rhythms by acting as an output molecule from the suprachiasmatic nucleus (Cheng et al., 2002), several studies have characterized the functions of PK2 in SCN target structures. In subfornical organ neurons, PK2 induces a reversible, dose-dependent depolarization and regulates voltage-activated potassium ($K^+$) channels (Cottrell et al., 2004). Later, these PK2-mediated effects were shown to be

modulated by the MAPK signaling pathway and sodium channels (Fry et al., 2008). In cultured dorsal nociceptive root ganglion (DRG) neurons, the amphibian PK2 homolog Bv8 increased the inward currents caused by heat and the capsaicin receptor, transient receptor potential vanilloid 1 (TRPV1). This effect was found to proceed by a PKC-epsilon-dependent signaling pathway (Vellani et al., 2006). Similarly, in rat trigeminal ganglion neurons involved in pain perception, treatment with PK2 reduced inward currents produced upon GABA(A) receptor activation. The inhibitory effect of PK2 on the GABA receptor could be blocked by the pan PKC inhibitor but not by the protein kinase A inhibitor H89 suggesting the involvement of one or more PKC isoforms.

Collectively, the studies to date demonstrate that diverse signaling pathways can be activated downstream of the prokineticin receptors by both PK ligands. This is exemplified by the fact that the prokineticin receptors are able to signal using Gi, Gq and Gs proteins. The functional cellular consequences of prokineticin signaling can be broadly categorized into cell survival, differentiation, migration, and excitability depending on the specific cell type and physiological function. Akt and ERK1/2 seem to be the main cell survival pathways activated by prokineticins as in the case of cardiomyocytes, endothelial cells, neural crest cells and myeloid cells. The same signaling pathways are also involved during angiogenesis and the survival of cancer cells including neuroblastomas and multiple myeloma cells. The ERK1/2 and other MAPK signaling pathways in cell differentiation induced by prokineticins and NFAT was identified as a key transcription factor downstream of the prokineticin receptors and regulates cytokine production. Nitric oxide generated by eNOS mediates the prokinetic functions of PK1 and PK2 on smooth muscle cells. MAPK and PKC signaling

have both been implicated in mediating the effects of PK-2 on excitable cells such as neurons of the subfornical organ and nociceptive dorsal root ganglion cells. It must be noted that although a wealth of evidence now supports a role for prokineticins as mediators of cell survival, to date neither of the prokineticins have been implicated in proapoptotic or cell death signaling pathways in any cell type.

**Prokineticin-2 signaling in the CNS**

Within the central nervous system, prokineticin-2 signaling through PKR2 has been shown to be the dominant ligand and receptor signaling axis for the prokineticins with PK1 being undetectable in most brain regions except in the nucleus tractus solitarus and lateral reticular nucleus of the hindbrain. The regional expression of PK2 and its two prokineticin receptors have been characterized in detail across the entire mouse brain by in situ hybridization (Cheng et al., 2006). The expression of PK-2 was also determined at high resolution using the GENSAT EGFP-PK2 transgenic mouse in which the expression of the EGFP reporter protein is driven by the PK2 promoter (Zhang et al., 2009). The brain was identified as the major site of PK2 expression when the prokineticins were first discovered (Li et al., 2001; Lin et al., 2002a; LeCouter et al., 2003). Subsequently, the major functions for PK2 signaling in the brain were elegantly demonstrated using knockout mice. Cheng et al. (2002) first identified PK2 as an output molecule from the SCN circadian clock and demonstrated that PK2 expression rhythmically oscillates in the SCN in response to light. PK2 also induced its own transcription in the SCN by PKR2 signaling through a positive feedback loop. The PKR2 receptor is expressed in various SCN output areas including the paraventricular hypothalamic nucleus (PVN), dorsal medial hypothalamic nucleus (DMH),

paraventricular thalamic nuclei (PVT). Mice lacking either PK2 or PKR2 were subsequently shown to have defective circadian rhythms by independent groups. Consistent with the expression pattern of PK2 in the SCN and the presence of its receptors in distal autonomic control centers of the brain, PK2 was shown to modulate the excitability of subsets of subfornical organ neurons in a MAPK-dependent manner, functionally validating a potential mechanism for regulation of autonomic functions. More recent studies also demonstrated the ability of PK2 to regulate neuronal excitability in area postrema (AP) neurons of the medulla to and thus modulate autonomic functions in the brain (Ingves and Ferguson, 2010).

**Table 2: Expression of PK2 and its receptors in different brain region**

| Brain Region | PK2 | PKR1 | PKR2 |
|---|---|---|---|
| **Olfactory bulb** | High | Moderate | High |
| **Cortex** | Not detected | Not detected | High |
| **Hippocampus & septum** | Not detected | Low | High |
| **Basal ganglia** | Moderate | Low | High |
| **Amygdala** | Not detected | Not detected | High |
| **Thalamus** | Not detected | Not detected | High |
| **Hypothalamus** | Low | Not detected | High |
| **Mesencephalon** | Not detected | Moderate | High |
| **Brainstem** | Not detected | Moderate | High |

References: Cheng et al.,( 2006) & Zhang et al., (2009)

Development and maintenance of the olfactory bulb has emerged as another crucial function for PK2 signaling in the brain that has now been confirmed by several independent research groups using both PK2 and PKR2 knockout mice (Ng et al., 2005; Matsumoto et al., 2006; Prosser et al., 2007; Zhang et al., 2007a). Secreted PK2 acts as a chemoattractant for neural progenitors that originate in the subventricular zone and continuously migrate into the olfactory bulb via the rostral migratory stream to generate new interneurons. PK2 knockout mice exhibit a significant reduction in both the size of the olfactory bulb and its architecture. Defective migration neuronal progenitors with their accumulation in the rostral migratory stream was also evident in these mice suggesting that incomplete development of the olfactory bulb originates from defective progenitor cell migration. The constitutive induction of PK2 in this region of the brain was found to be under the regulation of the proneural transcription factors Neurogenin 1 and MASH1,which bind at conserved E-box sites on the PK2 promoter to activate transcription (Zhang et al., 2007a). The effects of PK2 in the olfactory bulb and subventricular zone are most likely mediated by PKR2 signaling since PKR1 expression is essentially absent along this pathway. Further, PKR2 was found to be expressed by migrating neuroblasts and subventricular zone transient-amplifying progenitors in the adult mouse brain (Puverel et al., 2009). Along the same lines, PK2 expression was shown to be associated with areas of active neuronal migration and proliferation in the adult zebrafish brain during normal development and during post-injury telencephalon regeneration. (Ayari et al., 2010).

Emerging evidence suggests that PK2 is likely to have other important functions at distinct regions in the adult mammalian brain. The constitutive expression of the prokineticin

receptor PKR2 at high density across the brain even in the absence of either of its ligands suggest that PK2 might be induced locally at specific regions in the brain. Preliminary studies have shown that Bv8, the amphibian homologue of the prokineticins can protect cerebellar granule neurons and cortical neurons in culture against excitotoxic injury and degeneration, although in vivo evidence is lacking. Bv8 activated the typical PK2 survival signaling pathways Akt and p44/42 MAPK in cultured neurons to protect against excitotoxicity in vitro. Prokineticin receptors were also found to be expressed on cultured astrocytes and microglial cells and PK2 signaling induced calcium mobilization and proliferation in primary astrocytes (Koyama et al., 2006). High levels of PKR1 were reported in glial cells suggesting that PKR1 might be the dominant prokineticin receptor in non-neuronal cells in the CNS. The functional effects of PK2 on glial cells remain to be determined in detail and could be particularly relevant given the important functions of microglia and astrocytes in neurodegeneration and development.

# CHAPTER II

## PROTEOLYTIC ACTIVATION OF PROAPOPTOTIC KINASE PKCδ IN DOPAMINERGIC NEURONS BY TNFα DEATH RECEPTOR SIGNALING AND NEUROINFLAMMATION

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**Abstract**

The mechanisms of progressive dopaminergic neuronal loss in Parkinson's disease (PD) remain poorly understood largely due to the complex etiology and multifactorial nature of disease pathogenesis. Several lines of evidence from human studies and experimental models over the last decade have identified neuroinflammation as a potential pathophysiological mechanism contributing to the progression of the disease. Tumor necrosis factor-α (TNF) has recently emerged as the primary neuroinflammatory mediator that can elicit dopaminergic cell death in PD. However, the signaling pathways by which TNF mediates dopaminergic cell death have not been elucidated. Herein, we report that TNF death receptor signaling in dopaminergic neuronal cells activates an isoform in the novel PKC family, PKCδ, by caspase-3 and caspase-8 dependent proteolytic cleavage via the extrinsic apoptotic pathway. Notably, TNFR1 neutralizing antibody or the soluble TNF receptor Etanercept almost completely blocked the TNFα-induced PKCδ proteolytic

activation suggesting the involvement of the canonical death receptor pathway in the kinase activation. The proteolytic activation of PKCδ was accompanied by translocation of the kinase to the nucleus. Inhibition of PKCδ signaling by siRNA or overexpression of a cleavage-resistant mutant protects against TNF-induced cell death. Further, primary dopaminergic neurons obtained from PKCδ knockout (-/-) mice are also protected against TNFα neurotoxicity. We also demonstrate proteolytic activation of PKCδ in the mouse substantia nigra in the neuroinflammatory LPS model. Collectively, these results identify proteolytic activation of PKCδ proapoptotic signaling as a crucial downstream effector of dopaminergic cell death induced by TNFα. These findings also provide a rational for therapeutically targeting PKCδ to mitigate progressive dopaminergic degeneration resulting from chronic neuroinflammatory processes.

**Introduction**

Parkinson's disease (PD) is a debilitating neurodegenerative movement disorder affecting around 2% of the population over the age of 60 (Moore et al., 2005; Schapira, 2009). The pathological hallmark of the disease is a selective loss of dopaminergic (DA) neurons in the substantia nigra of the midbrain resulting in a depletion of striatal dopamine that is clinically manifest as a range of motor and nonmotor deficits (Obeso et al., 2010). A prominent clinical feature of PD is a sustained neuroinflammatory response in the SNpc that is initiated early during dopaminergic degeneration, and still evident in postmortem PD patients with increased microgliosis, dystrophic astrocytes and lymphocyte infiltration (Hirsch and Hunot, 2009; Glass et al., 2010; Tansey and Goldberg, 2010). Compelling evidence over the last decade from animal models, in vitro studies and PD patients has

demonstrated that the protracted neuroinflammation that occurs in the SNpc can exacerbate the degeneration of vulnerable dopaminergic neurons, but the molecular mediators and mechanisms involved are still being established.

Tumor necrosis factor-α (TNF) has recently emerged as an important neuroinflammatory mediator linked to dopaminergic degeneration in PD. Increased levels of TNF are present in the SNpc and the CSF of postmortem PD patients, and recently identified genetic polymorphisms in the TNFα gene locus have been linked to the development of PD (Boka et al., 1994; Mogi et al., 1994; Nagatsu et al., 2000; Nishimura et al., 2001; Wu et al., 2007; Bialecka et al., 2008). Increased TNF is also well documented in rodent and nonhuman primate models of PD induced by neurotoxicants and in transgenic models (Mogi et al., 1999; Barcia et al., 2005). Significantly, ablation of TNF signaling using knockout mice for TNFα or its receptors has been shown to attenuate MPTP-induced dopaminergic degeneration (Sriram et al., 2002; Ferger et al., 2004). Further, genetic or pharmacological inhibition of soluble TNF signaling with dominant negative mutants is protective against 6-OHDA and LPS-induced dopaminergic degeneration in vivo (McCoy et al., 2006; McCoy et al., 2008). Conversely, the chronic expression of low levels of TNFα in the substantia nigra of rats using an adenoviral vector can induce progressive dopaminergic degeneration with delayed motor deficits (De Lella Ezcurra et al., 2010). Together, these independent studies strongly implicate TNF in the progressive loss of dopaminergic neurons in PD. However, the signaling mechanisms by which TNF can mediate dopaminergic degeneration have not been characterized.

Protein Kinase Cδ (PKCδ), a member of the novel PKC isoform family, is highly expressed by dopaminergic neurons in the SNpc, in primary cultures and by dopaminergic cell lines (Zhang et al., 2007b). Recent studies from our laboratory and others have shown that PKCδ is proteolytically activated by caspase-3 during dopaminergic cell death and that genetic or pharmacological targeting of PKCδ can protect against dopaminergic degeneration in PD models (Anantharam et al., 2002; Kaul et al., 2003; Zhang et al., 2007a; Hanrott et al., 2008). In this paradigm, increased oxidative stress in dopaminergic neurons results in proteolytic activation of PKCδ by caspase-3 downstream of the intrinsic mitochondrial apoptotic pathway. The proteolytic cleavage of PKCδ by caspase-3 persistently activates the kinase as an effector of dopaminergic cell death.

However, PKCδ activation by extrinsic neuroinflammatory mediators like TNF has not been studied and would be relevant to the progression of PD by neuroinflammatory mechanisms. Since TNF also activates caspase-3 by signaling through its death receptors, we tested if PKCδ is proteolytically activated during TNF-induced dopaminergic cell death and in the mouse substantia nigra (SN) by neuroinflammatory mechanisms in the LPS model of dopaminergic degeneration. Our findings herein demonstrate PKCδ as a key downstream target of TNFα death receptor signaling in dopaminergic neurons and identify a novel link between neuroinflammatory mechanisms and dopaminergic degeneration.

**Materials and methods**

**Chemicals and reagents.** RPMI, neurobasal medium, B27 supplement, fetal bovine serum, L-glutamine, Sytox assay dye, IR-dye tagged secondary antibodies, penicillin, and

streptomycin and other cell culture reagents were purchased from Invitrogen (Gaithersburg, MD). Recombinant rat TNF, LPS (*E. coli* 0111:B4) and cytosine arabinoside were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant murine TNF and the TNFR1 neutralizing antibody were from R&D Systems (Minneapolis, MN). Etanercept (Enbrel) was purchased from Amgen Inc. (Thousand Oaks, CA). Antibodies for rabbit PKCδ and caspase-8 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse TH antibody was purchased from Chemicon (Temecula, CA) and MAP-2 antibody from Cell Signaling Technologies (Beverly, MA). $^{32}$P-ATP was purchased from Perkin Elmer (Boston, MA) and the AMAXA Nucleofector kit from Lonza (Basel, Switzerland). Caspase assay substrates and inhibitors were purchased from MP Biomedicals (Solon, OH). The DNA fragmentation assay kit was purchased from Roche Applied Science and the Bradford protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA).

**Stereotaxic infusion of LPS into the mouse substantia nigra (SN).** A single dose LPS injection model that was previously described (Saijo et al., 2009) was used to induce delayed, progressive loss of dopaminergic neurons in the substantia nigra. Eight week old C57/BL/6 mice (n = 6 per group) were anesthetized with a mixture of ketamine-xylazine (100mg/kg, 10mg/kg) and carefully immobilized on a stereotaxic apparatus (Benchmark Angle One, Leica Microsystems). The skin above the skull was prepped with alcohol and an incision was made to expose the skull. A single burr hole was carefully drilled at the injection site for the right SN and ophthalmic gel was used protect the eyes. The stereotaxic coordinates for the injection site were anteroposterior (AP) -3.3 mm, mediolateral (ML) -1.2 mm, dorsoventral (DV) -4.6 mm from bregma (Franklin and Paxinos, 2008) A stainless steel cannula attached

to a 5 µl Hamilton syringe was carefully inserted into the hole drilled at the injection site and a single dose of 5 µg LPS in a 1 µl volume was injected at the rate of 0.5 µl per minute. The needle was left in place for another 5 minutes to prevent retrograde flow of liquid along the needle track. Control mice were injected in an identical manner with equal volumes of saline. After surgery, the skin was sutured and carefully held in place using sterile, non-pyrogenic stainless steel clips (Autoclip Wound Closing System, Braintree Scientific, Inc). Mice were allowed to recover on a heating pad (Braintree Scientific, Inc) and were carefully monitored through recovery from anesthesia. All animal procedures were approved by the Iowa State University Institutional Animal Care and Use Committee (IACUC).

**Culture and treatment paradigm for rat dopaminergic N27 cells**. N27 cells were cultured as described previously (Clarkson et al., 1998; Kaul et al., 2003; Zhang et al., 2007a). Briefly, cells were cultured in RPMI 1640 medium containing 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 µg/ml). Cells were maintained in a humidified atmosphere of 5% $CO_2$ at 37°C. For treatment with TNF, RPMI medium containing 2% fetal bovine serum was used. Cells were washed twice in 2% serum RPMI and then treated with the indicated doses of recombinant rat TNF in 2% serum medium.

**Primary mouse mesencephalic neuron cultures.** Primary neurons were cultured from ventral mesencephalon tissue of gestational 14-day (E14) mouse embryos as described previously (McGuire et al., 2001; Zhang et al., 2007a) with some modifications. The ventral mesencephalon was dissected under a microscope and collected in ice cold DMEM F-12 complete medium (DMEM-F12 supplemented with 10% heat-inactivated fetal bovine serum

(FBS), 50 U/mL penicillin, 50 µg/mL streptomycin, 2 mM L-glutamine, 100 µM non-essential amino acids, and 2 mM sodium pyruvate). The tissue was then dissociated using trypsin-EDTA (0.25%) for 15 minutes at 37°C. Trypsinization was stopped by adding an equal volume DMEM-F12 complete medium and dissociated tissue was washed in the same medium to remove residual trypsin. The DMEM-F12 medium was aspirated out and the tissue triturated in neurobasal medium containing the B-27 antioxidant supplement, 500 µM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. After a single cell suspension was obtained, cells were passed through a 70 µm nylon mesh cell strainer to remove tissue debris and aggregates. Cells were counted using a Beckman Coulter ViCell XR automated cell counter and then plated at an equal density ($0.8 \times 10^6$ cells per well) in 24 well plates containing coverslips precoated with poly-D-lysine (100µg/ml). Cultures were maintained in neurobasal medium with B-27 antioxidant supplements and cytosine arabinoside (5 µM) was added to inhibit glial proliferation. Cultures were grown in a humidified $CO_2$ incubator (5% $CO_2$, 37°C) and the medium was changed every 2 days. Approximately 4 to 5-day-old cultures were used for treatment. The neuronal Cultures were verified to be around 98% free of glial cells at the time of treatment by using immunocytochemistry for MAP2, GFAP and iba1 as markers of neurons, astrocytes and microglia respectively. For TNF treatment, cultures were switched to serum-free neurobasal medium without antioxidant supplements and treated for 48 h. Recombinant murine TNF (30ng/ml) was added at the beginning of the treatment and re-added again 24 h later. At the end of the treatment, primary cultures were processed for TH immunocytochemistry and neurotransmitter uptake assays.

**DNA fragmentation and Sytox assays**. DNA fragmentation was quantified using the Cell Death Detection ELISA Plus assay kit (Roche Applied Science) as described in our previous publications (Anantharam et al., 2002; Kaul et al., 2003; Zhang et al., 2007a). This highly sensitive and reliable assay detects and quantifies early changes in apoptosis based on the amounts of histone-associated low molecular weight DNA released into the cytoplasm of cells. Briefly, N27 cells were plated in 6-well plates at 0.8 x $10^6$ cells/well and treated the next day with TNF for 16 h. Cells were collected using a cell scraper and centrifuged at 400 × g for 5 min. The cells were gently lysed using the lysis buffer provided with the kit. The lysate then was spun down again at 200 x g for 10 min to pellet the nuclear fraction and the supernatant was collected and used to measure DNA fragmentation according to the manufacturer's instructions for the ELISA protocol. The absorbance at 405 nm was measured against an ABTS solution as a blank (reference wavelength ~490 nm) using a Synergy-2 Multi-Mode Microplate Reader (BioTek Instruments, Inc). The raw absorbance values were normalized to the amount of protein present in the lysates. The Sytox cytotoxicity assay was performed using the Sytox green dye (Molecular Probes). The assay is based on the principle that live cells with intact plasma membranes exclude the dye that selectively enter cells with a compromised membrane and emits bright green fluorescence on binding to DNA (Sherer et al., 2002). N27 cells were grown in 24 well plates and 1 µM of the Sytox dye was added at the time of treatment. Cells were treated for 16 h with TNF and pretreated with Etanercept or the caspase-8 peptide inhibitor (IETD-FMK). The increase in green fluorescence as a result of TNF cytotoxicity was measured using a fluorescence microplate reader (Spectramax Gemini, Molecular Devices) at an excitation of 485 nm and 538 nm emission. Phase contrast

and fluorescence images of matching fields were taken on the same cells to qualitatively visualize the toxicity in N27 cells. Images were captures using a NIKON TE2000 microscope equipped with a SPOT digital camera (Diagnostic Inc)

**Caspase-3 enzymatic activity assays.** Enzymatic assays for caspase-3 activity were performed as described previously (Anantharam et al., 2002; Kaul et al., 2003) using acetyl-DEVD- amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC, 25 μm) as the fluorometric substrate for the reaction. The fluorescence signals generated upon cleavage of the AFC peptide substrate by caspase-3 was measured at 510 nm with an excitation of 400nm using a Synergy-2 Multi-Mode microplate reader. Protein concentrations were determined using the Bradford assay and used to normalize caspase activity.

**Confocal immunofluorescence microscopy for PKCδ translocation**. N27 cells were plated on poly-D-lysine (100 μg/ml) coated coverslips in 24 well plates at 0.2 x $10^6$ cells per well. The next day, the cells were treated with 30ng/ml of TNF for 6 h in 2% serum RPMI medium. Cells were fixed in 4% paraformaldehyde for 20 min and washed 5 times in PBS. The cells were blocked and permeabilized with blocking buffer (5% goat serum, 0.2% Triton X, and 0.05% Tween-20 in PBS) for 1 h and then incubated overnight at 4°C with a rabbit polyclonal PKCδ antibody (1:1000) that recognizes a C-terminal epitope present in both the native and the proteolytically cleaved protein. The cells were then washed 5 times in PBS and then incubated with secondary antibody (1:2000, Alexa 488 goat anti-rabbit) for 1 h at room temperature. Negative controls for non-specific staining that contained secondary antibody alone were used on parallel wells to ensure specificity of the fluorescent signals obtained. The cells were then washed 5 times in PBS and the nucleus was labeled using the

TOPRO 3 dye. The cells were then washed 3 more times in PBS and coverslips were mounted using the ProLong Gold antifade reagent (Molecular Probes). Confocal images were acquired with a Nikon EZ-C1 confocal system using the 488nm and 633 nm lasers to visualize PKCδ and TOPRO 3 respectively.

**Western blotting.** Lysates from N27 cells and brain tissue were prepared using a modified RIPA buffer and normalized for equal amounts of protein using the BCA protein assay (Pierce Biotechnology). Gel loading dye was added to the lysates and stored in aliquots at -80°C. Equal amounts of protein (30 to 50 µg) were loaded for each sample and separated on either 12% or 15% SDS-PAGE gels depending on the molecular weight of the target protein. After separation, the proteins were transferred to nitrocellulose membranes and non-specific binding sites were blocked by incubating the membranes in fluorescent Western blocking buffer (Rockland Immunochemicals) for 1 h and then probed with primary antibodies overnight at 4°C. Primary antibodies used were rabbit polyclonal PKCδ (1:500), mouse monoclonal TH (1:2000), rabbit polyclonal caspase-8 (1:200) and rabbit monoclonal cleaved caspase-3 (1:1000). Beta Actin (1:5000) was used as the loading control. After incubation, membranes were washed three times with PBS containing 0.05% Tween (PBST) and IR-dye tagged secondary antibodies (1:5000 Molecular Probes) were added and the membranes visualized on the Odyssey infra-red imaging system.

**PKCδ immunoprecipitation-kinase assays.** The PKCδ enzymatic kinase activity assay was performed as described previously (Anantharam et al., 2002; Kaul et al., 2005; Zhang et al., 2007a). Briefly, N27 cells or substantia nigra tissues were washed in ice cold PBS and then resuspended a mild RIPA lysis buffer containing protease and phosphatase inhibitor cocktail

(Pierce Biotechnology). The lysates were placed on ice for 20 min, sonicated gently and centrifuged at 13,000 rpm for 45 min. The supernatant was collected and protein concentration was determined using the Bradford assay (Bradford, 1976). All samples were made up to a concentration of 2 µg/ml and 500 µg of total protein in a 250 µl volume was immunoprecipitated overnight at 4°C using 5 µg of the PKCδ antibody. The next day, the protein A-agarose beads (Sigma-Aldrich) was added and the samples incubated for 1 h at room temperature. The protein A-bound antibody complexes were then washed three times in 2X kinase assay buffer (40 mM Tris, pH 7.4, 20 mM $MgCl_2$, 20 µM ATP, and 2.5 mM $CaCl_2$), and then resuspended in 40 µl of the same buffer. The kinase reaction was started by adding 40 µl of the reaction buffer containing 0.4 mg of histone H1, 50 µg/ml phosphatidylserine, 4 µM dioleoylglycerol, and 10 µCi of [$\gamma$-$^{32}$P] ATP at 3000 Ci/mM to the immunoprecipitated samples. The samples were incubated for 10 min at 30°C. The kinase reaction was stopped by adding 2X SDS loading buffer and boiling the samples for 5 min. The proteins were separated on a 12% SDS-PAGE gel and the phosphorylated histone H1 bands were scanned using a Fujifilm FLA 5000 imager. Image analysis and band quantification was performed using the Fujifilm Multigauge software package.

**Transfection of PKCδ siRNA and cleavage-resistant (PKCδ$^{D327A}$-CRM) mutant.** Design and synthesis of PKCδ siRNA is described in our previous publications (Yang et al., 2004; Zhang et al., 2007a). N27 cells were transfected with 40 to 50nM of either PKCδ or scramble siRNA duplexes using the AMAXA Nucleofector kit according to the manufacturer's instructions. Transfected cells were counted using a Vi-Cell XR automated cell counter and seeded at equal density (0.5 x $10^6$ cells/well) into 6-well plates. The cells were treated 24 h

after transfection to allow for optimal knockdown of gene expression, which was verified by Western blotting (Fig. 6B). At the end of the treatment, cells were collected and used for the DNA fragmentation assay as described above. For studies with the PKCδ cleavage-resistant mutant (PKCδ$^{D327A}$-CRM), stable N27 cell lines overexpressing either the mutant PKCδ$^{D327A}$ protein or the β-galactosidase (Lac Z) protein as the vector control were prepared as described in our publications (Latchoumycandane et al., 2005). Both cell lines were cultured in the presence of blasticidin for 3 passages before treatment. Stable expression of the PKCδ CRM-mutant and the Lac Z vector control at the time of treatment were confirmed by staining for the V5-epitope (Fig. 6D). Cells were plated at equal density (0.7 x $10^6$ cells per well) in 6 well plates and allowed to grow overnight. The next day, cells were treated with 30ng/ml of TNF for 16 h and processed for the DNA fragmentation assay.

**Dopamine and GABA uptake assays.** Uptake assays for tritiated dopamine and GABA on primary EVM cultures was performed according to previously published protocols (Liu et al., 2000a; Gao et al., 2002; McCoy et al., 2006). After treatment, the primary neuron cultures from PKCδ wild type (+/+) and knockout (-/-) mice were washed three times with 0.5 ml of warm Krebs–Ringer buffer (16 mM sodium phosphate, 120 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl2, 1.2 mM MgSO4, 1.3 mM EDTA, and 5.6 mM glucose; pH 7.4). The cultures were then incubated with either 5 μM [$^3$H]-Dopamine (30 Ci/mmol) and 10 μM [$^3$H]-GABA (90 Ci/mmol) in Krebs-Ringer buffer at 37°C for 20 min. The cells were then washed three times with ice cold Krebs-Ringer buffer and collected in 1 ml of 1N NaOH. Scintillation fluid was added to a total volume of 5 ml and the radioactivity counts were measured using a Tri-Carb Liquid Scintillation counter (Packard). In parallel wells, the nonspecific uptake of Dopamine

and GABA was determined by incubating with 10μM mazindol and 2 mM β-alanine respectively. The nonspecific uptake values were subtracted to obtain the data for high affinity neurotransmitter uptake.

**TH-positive cell counts and immunofluorescence.** After TNF treatment, primary neuron cultures from PKCδ wild type (+/+) and PKCδ knockout (-/-) mice were fixed in 4% paraformaldehyde and permeabilized with PBS containing 2% BSA, 0.2% Triton X-100 and 0.05% Tween-20. Blocking buffer (PBS with 2% BSA) was added for 1 h at room temperature and primary antibodies for TH (1:1000) and MAP2 (1:1000) were diluted in blocking buffer and incubated overnight at 4°C. The next day, cells were washed in PBS 4 times and incubated with appropriate Alexa-dye conjugated secondary antibodies for 1 h at room temperature. After several washes, the samples were counterstained with Hoechst to label the nucleus and coverslips were mounted using the Prolong Antifade (Molecular Probes) mounting medium. Images were acquired using a Nikon inverted fluorescence microscope (Model TE-2000U) equipped with a SPOT digital camera system (Diagnostic Instruments, Sterling Heights, MI). Image analysis was performed using the Metamorph software package (Universal imaging Systems, PA).

**Data analysis.** Data analysis was performed using Prism 4.0 software package (GraphPad Software, San Diego, CA). The data was first analyzed using one-way ANOVA and then Bonferroni's post-test was performed to compare all treatment groups. Differences of $p<0.05$ were considered statistically significant. The Student's t-test was used when differences between two groups were being compared.

**Results**

**TNF is toxic to rat N27 dopaminergic neuronal Cells.**

In order to test if TNF could induce dopaminergic cell death and determine the mechanism of toxicity, we treated rat dopaminergic N27 cells with recombinant TNF and determined the kinetics of cell death using the DNA fragmentation assay and increases in Sytox fluorescence. Exposure to TNF (30ng/ml) for 16 h significantly increased Sytox fluorescence and caused a threefold increase in DNA fragmentation compared to untreated controls (Fig. 1). Pretreatment with the TNF neutralizing drug Etanercept or a caspase-8 inhibitor (IETD-FMK) almost completely blocked TNF-induced apoptosis, indicating that canonical TNF death receptor signaling through activation of caspase-8 could be a potential mechanism for dopaminergic neurotoxicity caused by sustained exposure to TNF. In pilot experiments, we tested various doses of TNFα and treatment times and the 30 ng/ml dose was chosen for mechanistic studies with cell culture models although doses as low as 10ng/ml could induce toxicity. (Data not shown)

**Caspase-8 and caspase-3 are activated during TNF-induced cell death in dopaminergic cells.**

To further establish the mechanism of TNF toxicity on dopaminergic cells and the signaling pathways involved, we studied caspase activation downstream of TNFα signaling. High levels of the pro-caspase 8 protein were detected in N27 cells and TNF treatment induced dose dependent activation of caspase-8 at 3 h with increased accumulation of the active p20 fragment (Fig. 2A). Further, the activation of caspase-8 was attenuated by pretreatment with the TNFα inhibitor Etanercept (Fig. 2B). Since caspase-3 is an important

effector caspase downstream of TNFα death receptor signaling and is known to be activated in PD models and in the human PD brain (Hartmann et al., 2000; Zhao et al., 2001) , we studied caspase-3 activity in TNF-treated N27 cells. We found that TNF treatment for 6 h caused activation of caspase-3 which could be blocked by pretreatment with Etanercept and the caspase-8 inhibitor IETD-FMK (Fig. 2B, 2C). These results indicate that caspase-3 is activated in a caspase-8 dependent manner downstream of TNF death receptor signaling in N27 dopaminergic cells and could be an important mechanism linking the extrinsic neuroinflammatory insults to dopaminergic degeneration.

**TNFα induces proteolytic activation of Protein Kinase Cδ in N27 dopaminergic neuronal cells**

PKCδ has been shown to be an oxidative stress-sensitive kinase that is highly expressed in dopaminergic neurons. During oxidative stress-induced apoptosis in dopaminergic neurons and other cell types, PKCδ has been shown to be activated by caspase-3 mediated proteolytic cleavage at its hinge region resulting in persistent activation of the kinase and amplification of apoptotic signals. However, PKCδ activation by the extrinsic apoptotic pathway in dopaminergic neurons or its role in neuroinflammation-induced dopaminergic neurotoxicity has not been examined. We tested if PKCδ can be activated by caspase-3 during TNF-induced death of dopaminergic neuronal cells and function as a downstream apoptotic effector. Interestingly, TNF treatment caused a time and dose-dependent proteolytic activation of PKCδ that was detectable 3 h after treatment and increased strongly at 6 h (Fig. 3A). Dose response studies at the 6 h time point showed that doses as low as 10ng/ml of TNF could induce detectable proteolytic activation of PKCδ,

which increased substantially with 30ng/ml TNF. Higher doses of TNF (60ng/ml) did not significantly increase PKCδ cleavage further (Fig. 3B). Since caspase-8 and caspase-3 were activated by TNF in these cells, we tested if PKCδ proteolytic activation was dependent on this signaling pathway by using the specific caspase inhibitors DEVD-FMK and IETD-FMK. Pretreatment with the caspase-8 and caspase-3 peptide inhibitors suppressed TNF-induced proteolytic activation of protein kinase Cδ (Fig. 3C). Collectively, these results demonstrate for the first time that the pro-apoptotic kinase PKCδ is activated by caspase-8 and caspase-3 dependent proteolytic cleavage during TNF-induced apoptosis in dopaminergic neuronal cells.

**Proteolytic activation of PKCδ by TNF signaling persistently activates the kinase in N27 dopaminergic neuronal cells**

Unlike many other proteins for which proteolytic cleavage can result in loss of function or inactivation, the proteolytic cleavage of PKCδ by caspase-3 during oxidative stress-induced dopaminergic cell death has been shown to persistently activate this kinase (Anantharam et al., 2002; Kanthasamy et al., 2003; Kaul et al., 2003; Zhang et al., 2007a). We used immunoprecipitation-kinase assays and Western blotting to verify if TNFα-induced proteolytic activation of PKCδ leads to sustained activation of the kinase in dopaminergic N27 cells. As shown in Fig. 4A treatment with TNF for 6 h significantly increased proteolytic cleavage of PKCδ, which was blocked by pretreatment with Etanercept or a TNFR1 (TNFRSF1) receptor-neutralizing antibody. PKCδ IP-kinase assays performed on the same lysates showed a robust increase in PKCδ kinase activity in TNF-treated samples, which could be attenuated by pretreatment with Etanercept or by TNFR1 (TNFRSF1)

receptor neutralization. Together, these findings demonstrate that PKCδ is proteolytically activated in N27 dopaminergic neuronal cells by a TNFR1-dependent signaling pathway, resulting in sustained activation of the kinase during TNF-induced cell death.

**PKCδ translocates to the nucleus during TNFα-induced apoptosis.**

Rapid and dynamic translocation to distinct subcellular locations following activation is a characteristic feature of PKCδ in different cell types and typically delineates the unique functional role of this kinase in a particular system (Gomel et al., 2007). PKCδ has been shown to translocate to the plasma membrane, the endoplasmic reticulum, the mitochondria and the nucleus in a cell- and stimulus-specific manner. Unique substrate proteins have been identified as candidates for PKCδ phosphorylation in these subcellular locations that participate in a range of cellular functions from apoptosis to cell migration and activation of immune cells (Steinberg, 2004). Particularly, nuclear translocation of PKCδ has been shown to occur following proteolytic activation by apoptotic stimuli. A nuclear localization signal (NLS) is present on the C-terminus of the catalytic domain that is released upon proteolytic cleavage by caspase-3. To further characterize the functional role of PKCδ in dopaminergic cell death induced by TNF, we used immunocytochemistry and confocal microscopy to study the subcellular localization of PKCδ following TNF treatment. N27 cells were treated with TNF for 6 h, the time point at which the highest level of cleaved PKCδ was detected, and confocal immunofluorescence was used to visualize the subcellular localization of PKCδ. We detected prominent nuclear localization of PKCδ after 6 h of TNF treatment, while in untreated controls PKCδ was almost completely localized in the cytoplasm (Fig.5A). Interestingly, we also observed punctuate staining of PKCδ at distinct locations around the

perinuclear region in TNF-treated cells (labeled with red arrows) in addition to the overall increase in nuclear localization. The nuclear translocation of PKCδ points to a pro-apoptotic function for this kinase during TNF death receptor signaling in these cells. The distinct pattern of perinuclear accumulation, however, appears to be unique to TNF signaling in these cells and does not occur during treatment with other neurotoxicants we have studied previously that induce apoptosis by the intrinsic cell death pathway.

**Overexpression of a cleavage-resistant mutant (PKCδ$^{D327A}$-CRM) or siRNA knockdown of PKCδ protects against TNFα dopaminergic cell death.**

The proteolytic activation of PKCδ by caspase-3 and its translocation to the nucleus indicate an important function for this kinase in effecting apoptosis induced by TNF in dopaminergic N27 cells. To validate a pro-apoptotic role for PKCδ signaling in this system, we used siRNA suppression of PKCδ and a caspase-3 cleavage-resistant mutant of PKCδ (PKCδ$^{D327A}$-CRM). N27 cells were transfected with siRNA to PKCδ or scramble siRNA and incubated for 24 h to allow for maximum suppression of PKCδ protein levels, which we validated by Western blotting (Fig. 6B). The transfected cells were then treated with TNF for 16 h and processed for the DNA fragmentation assay. In N27 cells transfected with scramble siRNA, TNF caused a significant increase in DNA fragmentation while the PKCδ siRNA transfected cells were protected from cell death (Fig. 6A). These results confirm that PKCδ is required for TNF-induced apoptosis in dopaminergic cells and that genetic or pharmacological modulation of PKCδ may be effective in attenuating TNF-induced neurotoxicity. To determine the relevance of PKCδ proteolytic cleavage during pro-apoptotic TNF signaling, we used N27 cells over expressing a mutant PKCδ protein that is

resistant to proteolytic cleavage (PKCδ$^{D327A}$-CRM) by caspase-3 due to a point mutation at the cleavage site. Stable N27 cells that expressed either the cleavage-resistant PKCδ mutant or the LacZ protein were established as described previously (Latchoumycandane et al., 2005). The cells were treated with TNF for 16 h and processed for DNA fragmentation. As shown in Fig.6C, TNF treatment caused a 2.5-fold increase in DNA fragmentation in LacZ cells, while cells expressing the PKCδ$^{D327A}$-CRM mutant were protected from TNF toxicity, indicating that proteolytic cleavage of PKCδ is crucial for TNF-induced cell death. Collectively, these results demonstrate that PKCδ functions as a proapoptotic kinase during TNF-induced dopaminergic neurotoxicity and that proteolytic cleavage of PKCδ by caspase-3 downstream of TNF signaling is required for cell death.

**Primary dopaminergic neurons obtained from PKCδ knockout (-/-) mice are protected from TNFα neurotoxicity**

In order to extend our results from the mechanistic studies with dopaminergic N27 cells to a more relevant system, we used primary mouse ventral mesencephalic dopaminergic neuron cultures to study the role of PKCδ in mediating TNF-induced dopaminergic neurodegeneration. We established primary EVM neuronal (>95% glial-free) cultures from PKCδ wild type (+/+) and knockout (-/-) mice and treated them with recombinant TNF in supplement-free neurobasal medium, as described in the Methods section. Dopaminergic neurotoxicity was assessed by dopamine uptake assays and by TH-positive cell counting. As shown (Fig.7A,B), TNF exposure caused a 40-50% decrease in dopamine uptake and TH-positive cell counts in cultures from PKCδ WT (+/+) mice, consistent with previous reports of dopaminergic neurotoxicity induced by TNF in similar model systems (McGuire et al.,

2001; McCoy et al., 2006). Significantly, in primary neuron cultures obtained from PKCδ knockout (-/-) mice, TNF induced only about a 20% reduction in dopamine uptake and TH-positive neuron counts, indicating that these cultures were resistant to TNF toxicity. With TH as a marker for dopaminergic neurons and MAP-2 as a pan-neuronal marker, we utilized double labeling immunofluorescence to visualize the effects of TNF exposure on the dopaminergic and other neuron populations in primary EVM cultures. Untreated cultures from both PKCδ wild type (+/+) and knockout (-/-) mice had similar numbers of dopaminergic neurons and displayed extensive branched neurites and normal cell bodies (Fig. 7D, top panel). In TNF-treated cultures, fewer TH-positive neurons with smaller cell bodies and a clear loss of neurites was evident in cultures from PKCδ wild type (+/+) mice. In contrast, dopaminergic neurons in cultures from PKCδ knockout (-/-) mice displayed visible branched neurites and more numerous cell bodies with normal morphology (Fig. 7D, lower panel) demonstrating significant protection against TNF-toxicity in these cultures. These results are consistent with the established function of PKCδ as a common apoptotic effector in different cell types (Humphries et al., 2006). We also used GABA uptake assays on cultures treated with TNF under the same conditions to determine if TNF neurotoxicity extended to other neuronal populations relevant to PD such as the GABA neurons. In agreement with findings from previous studies (McCoy et al., 2006), TNF treatment did not induce a significant reduction in GABA uptake under our treatment conditions, even when higher doses (60ng/ml) were used (Fig. 7C).

**PKCδ is activated by proteolytic cleavage in the mouse SNpc in the LPS neuroinflammatory model of Parkinson's disease.**

Next we sought to extend the significance of our results obtained with the N27 cell culture model and primary neuron cultures to an in vivo model of dopaminergic degeneration relevant to neuroinflammation. Elevated levels of TNFα and neuroinflammation are well documented in the established MPTP and 6-OHDA models of PD. In fact, the use of dominant negative TNFα has been shown to be neuroprotective against 6-OHDA-mediated dopaminergic neurotoxicity (McCoy et al., 2006). However, previous studies from our laboratory and others have demonstrated proteolytic activation of PKCδ downstream of the intrinsic mitochondrial apoptotic pathway initiated by oxidative stress (Kanthasamy et al., 2003; Kaul et al., 2003; Zhang et al., 2007a; Hanrott et al., 2008). Since this could potentially confound our studies, which were study the proteolytic activation of PKCδ by extrinsic neuroinflammatory mechanisms, we avoided the use of the MPTP or 6-OHDA models of dopaminergic degeneration. Instead, we selected the widely used stereotaxic LPS injection model of neuroinflammation-induced dopaminergic degeneration for our experiments (Herrera et al., 2000; Liu et al., 2000b; McCoy et al., 2006; Saijo et al., 2009). In this model, a single injection of LPS into the rodent substrantia nigra elicits a sustained, localized neuroinflammatory response that results in a delayed and selective loss of dopaminergic neurons in the SNpc. The dopaminergic degeneration usually starts within days of the LPS injection and is complete around two weeks (Liu et al., 2000b). Significantly, dopaminergic degeneration is evident up to a year later and appears to be permanent in this model (Herrera et al., 2000). C57bl6 mice were stereotaxically injected with a single dose of 5 µg LPS into

the substantia nigra and sacrificed 14 days later, the time at which significant dopaminergic degeneration occurs in this model(Saijo et al., 2009). The SN tissue was dissected and probed for proteolytic activation of PKCδ using Western blotting and IP-kinase assays as described in our previous publications (Zhang et al., 2007a). As shown in Fig. 8B, mice that received saline injections did not show proteolytic activation of PKCδ in the SN, while in LPS-injected mice, strong proteolytic activation of PKCδ was evident at 14 days. The PKCδ Western blots were reprobed for tyrosine hydroxylase to confirm accurate dissection of the substantia nigra region. As expected, the LPS-injected mice had decreased TH protein levels (Fig. 8B), indicative of the increased loss of dopaminergic neurons. We also used PKCδ immunoprecipitation-kinase assays with a histone substrate on nigral tissue samples from LPS- and saline-injected mice. In nigral tissues from LPS-injected mice; we detected substantially higher PKCδ kinase activity compared to saline injected controls (Fig. 8A), demonstrating that the proteolytic cleavage of PKCδ resulted in activation of the kinase in vivo. Equal protein loading and accurate dissection of the nigral region was confirmed by Western blotting for β-actin and TH respectively. Together, these results demonstrate for the first time that protein kinase Cδ can be activated by proteolytic cleavage in the substantia nigra during dopaminergic degeneration caused by LPS induced neurotoxicity

**Discussion**

In this study, we identify PKCδ as a novel signaling mediator of TNFα-induced neurotoxicity in dopaminergic neurons and demonstrate that proteolytic activation of PKCδ regulates dopaminergic degeneration induced by TNF. We also demonstrate for the first time, the proteolytic activation of PKCδ in the mouse substantia nigra by neuroinflammatory

mechanisms during LPS-induced dopaminergic degeneration. Previous reports from our laboratory and other independent research groups have shown that proteolytic activation of PKCδ in dopaminergic neurons is a common effector of apoptosis v induced by diverse neurotoxic stimuli that converge on the intrinsic mitochondrial apoptotic pathway primarily through intracellular oxidative stress mechanisms (Anantharam et al., 2002; Kaul et al., 2003; Yang et al., 2004; Latchoumycandane et al., 2005; Kanthasamy et al., 2006; Zhang et al., 2007a; Hanrott et al., 2008; Sun et al., 2008; Cunningham et al., 2009). However, proteolytic activation of PKCδ by extrinsic apoptotic effectors has not been studied and would be relevant to the progressive loss of dopaminergic neurons resulting from chronic neuroinflammatory processes involving TNFα and other potentially neurotoxic mediators (Block et al., 2007; Whitton, 2007). Based on the substantial literature supporting a degenerative role for the TNFα during dopaminergic cell death in PD models, we hypothesized that PKCδ can be proteolytically activated downstream of canonical TNF death receptor signaling by caspase-3 in dopaminergic neurons. Since proteolytic cleavage of PKCδ typically results in persistent activation of the kinase as an apoptotic effector, we reasoned that blocking proapoptotic PKCδ signaling could protect against TNF toxicity.

The results from our mechanistic studies using N27 rat dopaminergic cells demonstrate for the first time that soluble TNF induces a time- and dose-dependent proteolytic cleavage of PKCδ (Fig. 3A,B) by a caspase-8 and caspase-3 signaling pathway (Fig. 3C), indicative of canonical TNF death receptor signaling via caspase-8. Using the anti-TNF drug Etanercept and neutralizing antibodies to the TNFR1 (p55) death receptor, we show that proteolytic cleavage of PKCδ by soluble TNF is dependent on TNFR1 signaling and is accompanied by

a substantial increase in PKCδ kinase activity (Fig. 4A, B), typical of proteolytic activation. The subcellular localization of PKCδ following activation has been shown to be an important factor determining the pro or anti-apoptotic functions of this kinase in different model systems (DeVries-Seimon et al., 2007; Gomel et al., 2007). In particular, numerous studies have demonstrated that nuclear translocation of PKCδ is required for induction of apoptosis by diverse stimuli and is driven by a bipartite nuclear localization sequence at it's C-terminus (DeVries-Seimon et al., 2007; Reyland, 2007; Humphries et al., 2008). On the other hand, proteins belonging to the TNF superfamily such as BAFF (B-cell-activating factor belonging to the TNF family) have been shown to prevent nuclear accumulation of PKCδ and thus prolong cell survival in B-cells (Mecklenbrauker et al., 2004). Our confocal immunofluorescence studies reveal a distinct localization of PKCδ to the nucleus 6 h after TNF treatment, the time point at which maximum proteolytic cleavage of PKCδ was evident (Fig. 3A). Interestingly, while intense PKCδ staining is evident throughout the nucleus in TNF-treated cells, there is also a distinct pattern of accumulation PKCδ at specific sites around the peri-nuclear region and within the nucleus (shown as an inverse gray scale image in Fig. 5B). In our experience, this pattern of PKCδ nuclear localization is unique to TNF treatment and is not evident during nuclear translocation induced by other neurotoxicants such as MPTP, 6-OHDA or hydrogen peroxide in these cells. It is likely that PKCδ has unique substrates in and around the nucleus and functions differently during cell death induced by extracellular signals such as TNF. The substrates of PKCδ and the functional significance of these findings are currently under investigation in our laboratory. The kinetics of PKCδ proteolytic activation and it's translocation to the nucleus in dopaminergic cells is

consistent with a proapoptotic role for PKCδ downstream of TNFα signaling in dopaminergic cell death. Indeed, suppressing total PKCδ protein levels using siRNA or blocking proteolytic activation using a PKCδ cleavage-resistant mutant (PKCδ$^{D327A}$-CRM) affords robust protection against TNF toxicity in N27 cells (Fig. 6A,B). The results from our mechanistic experiments are further supported by our studies in primary mouse EVM neuronal cultures treated with soluble TNF, a more relevant model system for dopaminergic degeneration compared to immortalized cell lines. While TNF toxicity on primary dopaminergic neurons was mild compared to other neurotoxicants such as $MPP^+$ (Zhang et al., 2007a), we observed a substantial reduction in TH neuron numbers (Fig. 7A) and functional impairment using dopamine uptake studies (Fig. 7B) in cultures from PKCδ wild type (+/+) mice, consistent with previous reports (McGuire et al., 2001; McCoy et al., 2006). In contrast, dopaminergic neurons in EVM cultures obtained from PKCδ knockout (-/-) mice were protected against TNF toxicity, indicating that PKCδ signaling is essential for dopaminergic degeneration by the extrinsic cell death cascade triggered by TNF.

Evidence from previous studies (McGuire et al., 2001; McCoy et al., 2006) and our own results here (Fig. 4A,B), indicate that TNF can induce dopaminergic neurotoxicity by a direct mechanism of action, possibly via the TNFR1 (TNFRSF1) death receptor, which has been shown to be highly expressed on these cells. However, several lines of evidence suggest that TNF can also contribute to dopaminergic degeneration indirectly by potentiating microglial activation and release of ROS or other neurotoxic mediators, that increase local oxidative stress levels in the SNpc (Sriram et al., 2006; Block et al., 2007; De Lella Ezcurra et al., 2010). Since TNF levels in the SNpc are elevated both in animal models and human

PD patients and given the concomitant microglial activation that is evident, it is likely that potentiation of microglial neurotoxic responses by TNF occurs in vivo, although it may not be the primary mechanism of dopaminergic neurotoxicity as shown by McCoy et al. (2006) using mixed neuron-glial cultures. With the plethora of important functions ascribed to TNF in the normal brain and given the pleotropic nature of this important cytokine, it is likely that neurotoxic TNF signaling is tightly regulated in the nigrostriatal system, and that TNF by itself may be insufficient to directly elicit dopaminergic degeneration in vivo, as shown by Castano et al. (2002). Rather, a chronic increase in TNF levels in the nigrostriatal system may drive the progressive loss of compromised, vulnerable dopaminergic neurons in the pro-oxidant environment of the SNpc, both by TNF apoptotic signaling and by potentiation of microglial neurotoxic responses, including ROS and RNS (McGuire et al., 2001; McCoy et al., 2006; Block et al., 2007; Qin et al., 2007; De Lella Ezcurra et al., 2010). Nonetheless, our data demonstrating that PKCδ is proteolytically activated in the SNpc in the purely neuroinflammatory LPS model (Fig.8A,B) of PD implicates proapoptotic PKCδ signaling as a common downstream effector of dopaminergic cell death triggered by convergent neuroinflammatory mechanisms involving TNF and other neurotoxic mediators in vivo.

Despite extensive research into the etiology of PD for several decades, a cogent basis for the selective vulnerability and substantial loss of SNpc dopaminergic neurons still remains elusive. The emerging consensus that PD results from complex interactions between environmental, genetic and cellular processes that induce dopaminergic cell death over time, (Sulzer, 2007; Obeso et al., 2010), highlights the multifactorial nature of the disease and underscores the importance of identifying therapeutic targets that are relevant across these

multiple mechanisms of disease pathogenesis. While oxidative stress, mitochondrial dysfunction and proteasomal impairment have been identified as causative factors that can trigger dopaminergic degeneration, reactive microgliosis and dysregulated neuroinflammation can sustain this process and result in progressive neurotoxicity (Block et al., 2007; Whitton, 2007; Glass et al., 2010). A wealth of evidence already supports a role for proteolytic activation and proapoptotic PKCδ signaling during dopaminergic cell death in PD models via proteasomal impairment, mitochondrial dysfunction and oxidative stress mechanisms. Further, pharmacological targeting of PKCδ with kinase or peptide inhibitors has shown efficacy in both in vitro and in vivo models of PD. (Kanthasamy et al., 2006; Zhang et al., 2007a). Our results from this study, provide exciting new evidence for PKCδ proapoptotic signaling during dopaminergic cell death induced by TNF and in LPS-induced dopaminergic degeneration in vivo by neuroinflammatory mechanisms. Together with previous studies, our results here suggest that proteolytic activation of PKCδ could be a common downstream target of multiple mediators of dopaminergic degeneration by both extrinsic and intrinsic cell death mechanisms, making it an attractive therapeutic target during the progressive phase of PD.

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**Figure 1.**

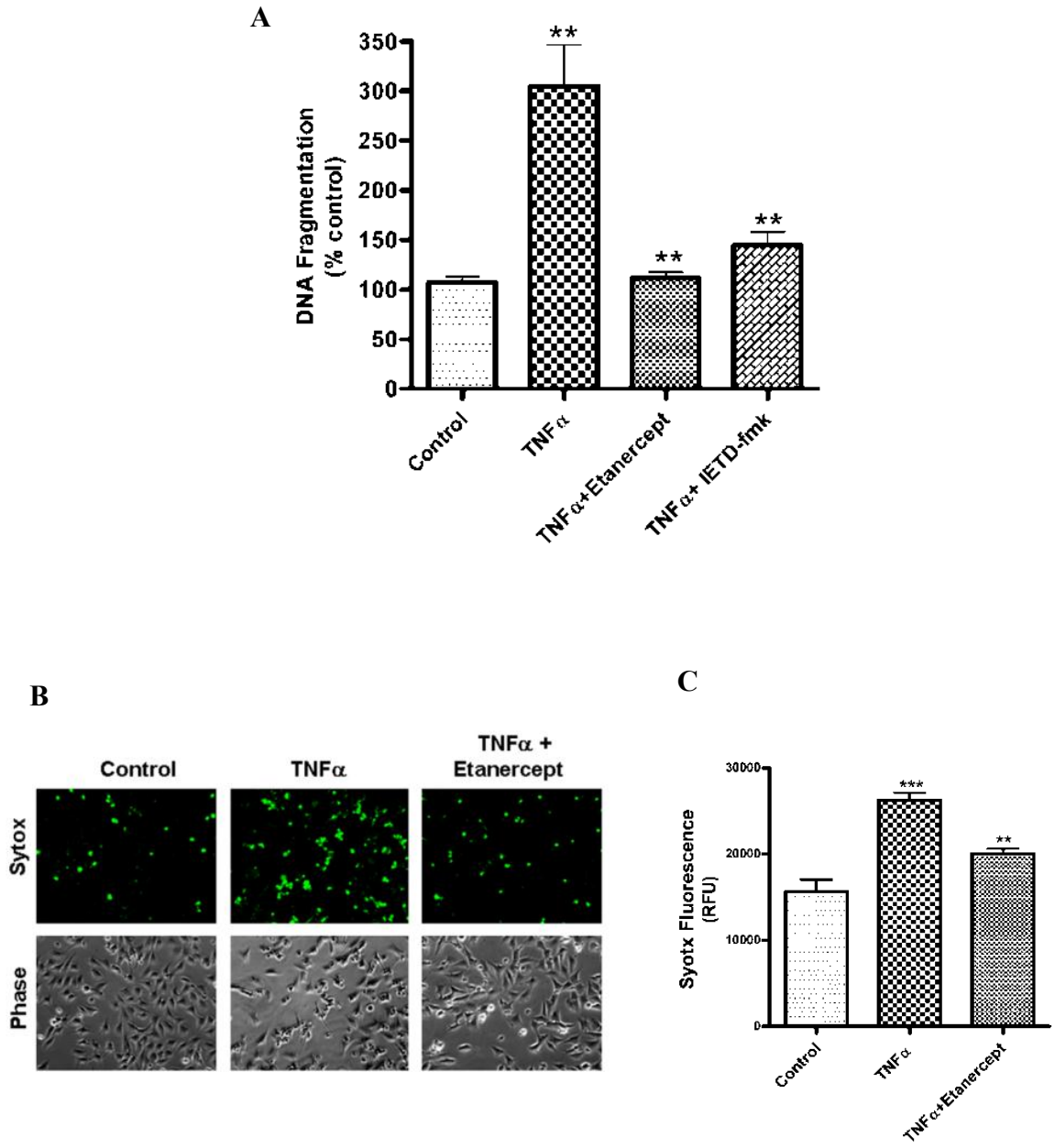
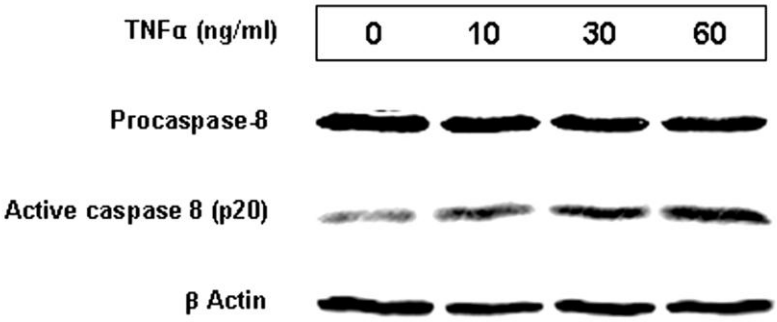



**Figure 1. TNF toxicity to N27 dopaminergic cells. (A) DNA fragmentation assay.** N27 dopaminergic cells were treated with TNF (30ng/ml) for 16 h or pretreated for 30 min with either Etanercept (5µg/ml) or the caspase-8 peptide inhibitor IETD-fmk (25µM), and processed for DNA fragmentation. Experiments were performed in triplicate; the raw values

were normalized using protein concentration and expressed as percent control. TNF treatment significantly increased DNA fragmentation in N27 cells, which was attenuated by Etanercept or caspase-8 inhibition. **(B)** Visualization of TNF toxicity by Sytox fluorescence. N27 cells treated with TNF (30ng/ml), or pretreated with Etanercept (5µg/ml) were processed for Sytox green imaging after 16 h. Phase contrast and fluorescence images were acquired on the same fields from live cells after 20 min of incubation with the Sytox dye. Increased green fluorescence is evident with TNF treatment, indicative of cytotoxicity. **(C)** Quantitative estimation of Sytox fluorescence. Sytox fluorescence was quantified by measuring the fluorescence intensity using a plate reader. Data represent the group mean $\pm$ SEM of six to eight individual readings. Asterisks (***$p<0.001$ and **$p<0.01$) indicate significant differences between control cells and TNF treatments, or between TNF-treated cells and those pretreated with Etanercept or IETD-fmk.

**Figure 2.**

**A**

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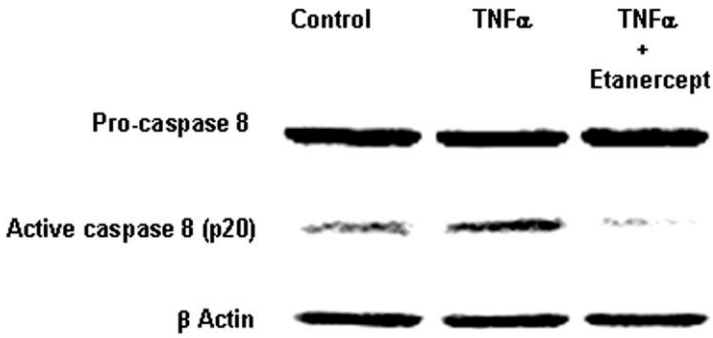


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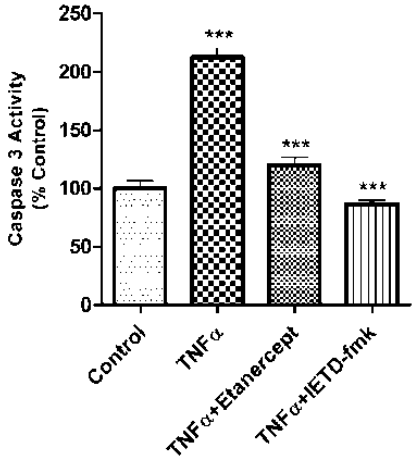


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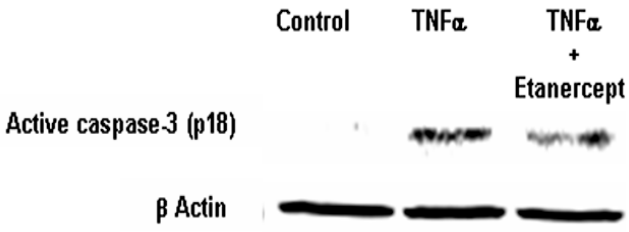


**Figure 2. Activation of caspase-8 and caspase-3 in N27 cells by TNF treatment**. **(A)** Western blot for active caspase-8. Lysates from N27 cells treated with different doses of TNF (10 to 30ng/ml) for 3 h were probed using a rabbit polyclonal antibody to caspase-8 that detects both procaspase-8 and the active form of the enzyme. TNF treatment induced a dose-dependent increase of the active caspase-8 (p20) fragment. **(B)** Etanercept blocks TNF-induced caspase-8 activation. Pretreatment with Etanercept (5µg/ml) for 30 minutes suppressed the activation of caspase-8 induced by TNF (30ng/ml) treatment in N27 cells. (C, D) Caspase-3 activity assay and western blot. Enzymatic assays for caspase-3 activity were performed using a fluorogenic peptide substrate (Ac-DEVD-AFC) on lysates from N27 cells treated with TNF (30ng/ml) for 6 h or pretreated with either Etanercept (5µg/ml) or the caspase-8 inhibitor IETD-fmk (25µM). Raw values were normalized using protein concentrations and expressed as percent control. TNF treatment induced significant activation of caspase-3 which was blocked by Etanercept and the caspase-8 inhibitor. Caspase-3 activation was also confirmed by western blotting (D). Data represent the group mean $\pm$ SEM of six to eight individual readings. Asterisks (***$p<0.001$) indicates significant differences between controls and TNF-treated cells or TNF treated cells and pretreatment groups with Etanercept and IETD-fmk.

**Figure 3.**

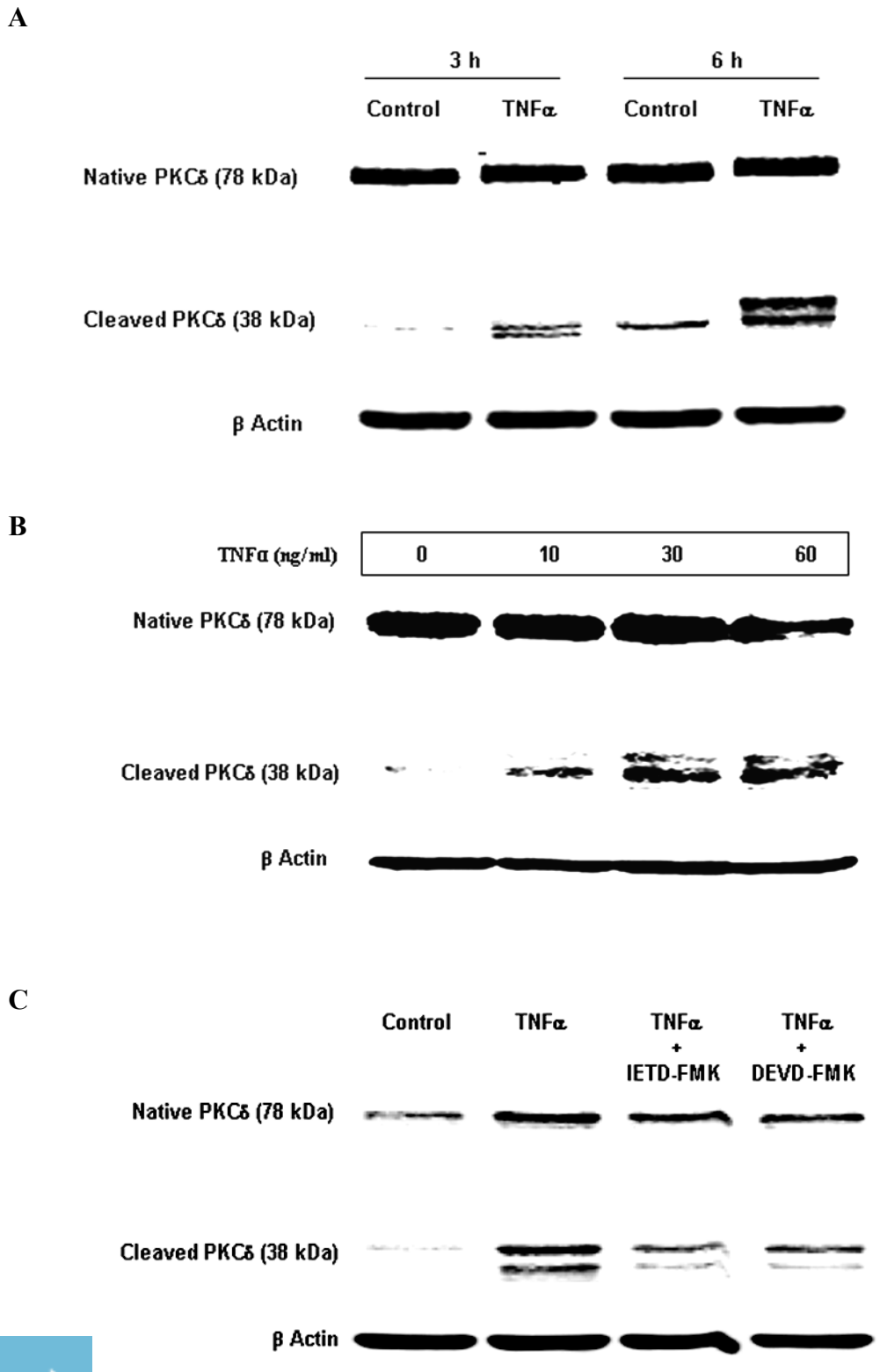
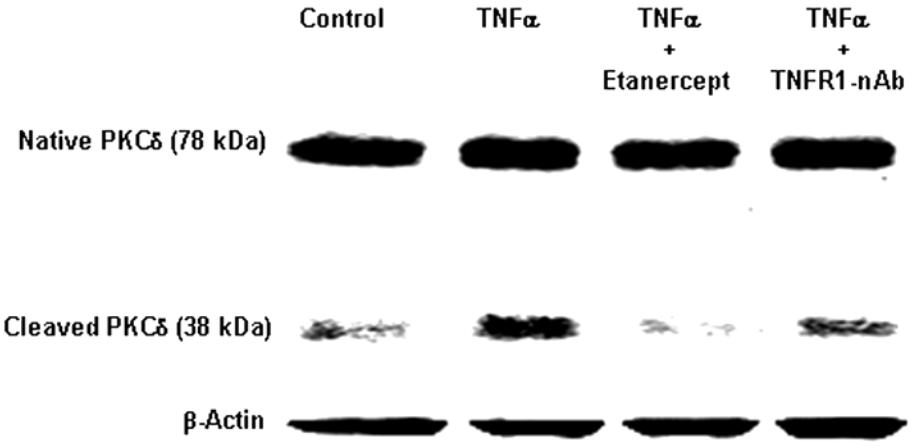


**Figure 3. TNF activates PKCδ by proteolytic cleavage downstream of caspase-3 and caspase-8. (A)** Time course of PKCδ activation. N27 cells were treated with recombinant TNF (30ng/ml) for 3 h and 6 h and lysates were probed by Western blotting with an antibody to the C-terminus that detects both the native protein (78 kDa) and the proteolytically cleaved catalytic fragment (38 kDa). Actin was used as the loading control. TNF treatment caused a time-dependent increase in PKCδ cleavage which peaked at 6 h. **(B)** Dose-dependent proteolytic activation of PKCδ by TNF treatment. N27 cells were treated with increasing doses of TNF (0 to 60ng/ml) for 6 h and lysates were probed for PKCδ protein levels by Western blotting. TNF induced a dose dependent increase in PKCδ cleavage stating at 10ng/ml. **(C)** Caspase-3 and caspase-8 dependent proteolytic cleavage of PKCδ. N27 cells were treated with 30ng/ml of TNF alone for 6 h or in the presence of peptide inhibitors (25μM) of caspase-8 (Ac-IETD-fmk) and caspase-3 (Ac-DEVD-fmk). Cells were collected and lysates were probed for PKCδ proteolytic cleavage by Western blotting. Proteolytic activation of PKCδ by TNF treatment was attenuated by inhibition of caspase-3 and caspase-8.

**Figure 4.**

**A**

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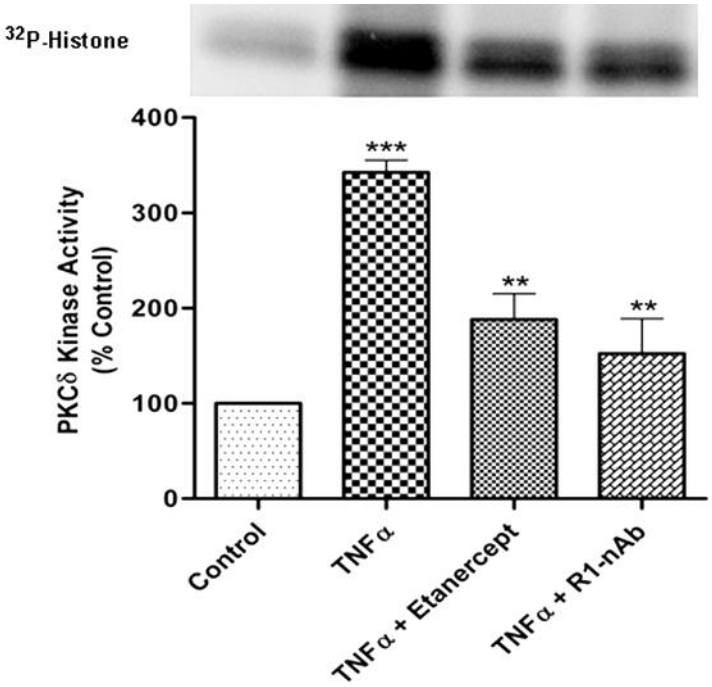
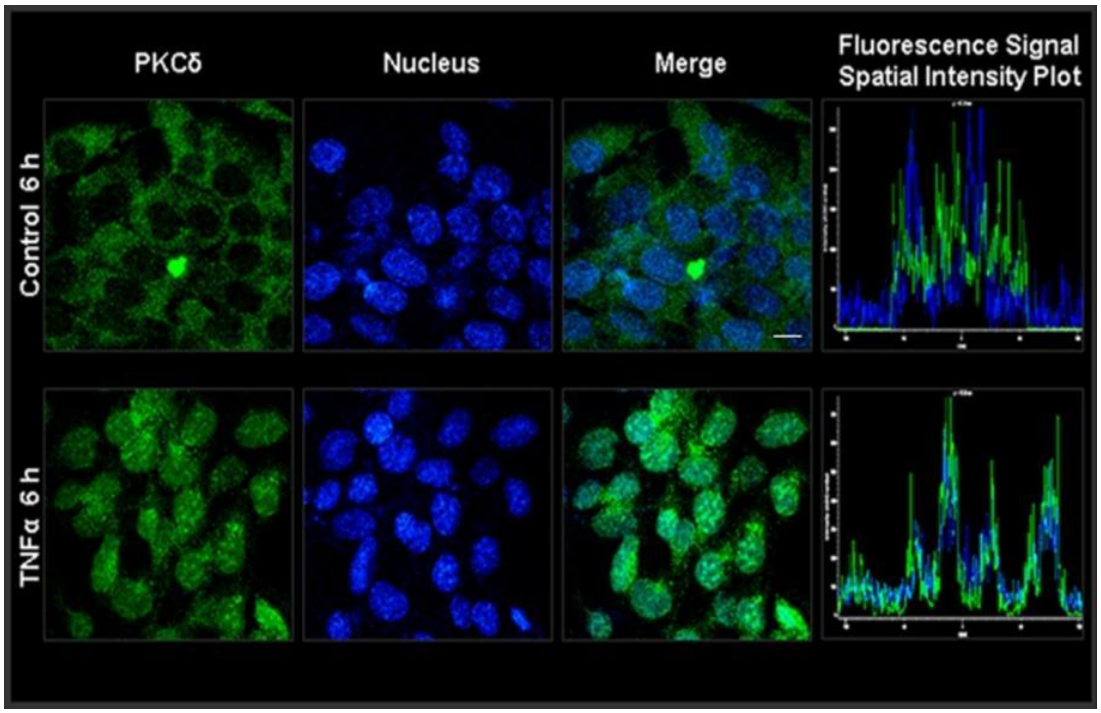


**Figure 4. Proteolytic cleavage of PKCδ downstream of TNF signaling persistently activates the kinase in dopaminergic N27 cells and is dependent on TNF-R1 (p55) signaling. (A)** Western blot of proteolytic activation of PKCδ in TNF-treated N27 cells. N27 cells were treated with TNF (30ng/ml) for 6 h or pretreated with either a TNFR1 neutralizing antibody (p55-nAb, 20 µg/ml) or Etanercept (5µg/ml) for 30 minutes and lysates were probed for PKCδ proteolytic cleavage by Western blotting. TNF induced strong proteolytic cleavage of PKCδ which could be blocked by neutralizing TNFR1 receptor signaling. (B) Immunoprecipitation-kinase assay for PKCδ activation. IP-kinase assays were performed on the same cell lysates used for Western blots. PKCδ was immunoprecipitated from 500µg of total protein from each sample and used for the in vitro kinase activity assay with a histone substrate and radiolabeled [$\gamma$-$^{32}$P] ATP. PKCδ activity was quantified using densitometric analysis of the $^{32}$P-histone band intensity and expressed as percent of control samples. As seen, the proteolytic cleavage of PKCδ induced by TNF treatment **(A)** is accompanied by a concomitant increase in kinase activity and is dependent on signaling through the p55 TNFR1 receptor **(B).** A representative kinase assay gel is shown. Data represent the group mean $\pm$ SEM of densitometric values obtained from three independent experiments. Asterisks (***$p<0.001$, ** $p<0.01$) indicate significant differences between control and TNF-treatment groups or between TNF treatment and pretreatment groups with Etanercept or TNFR1 neutralizing antibody (TNFR1-nAb).

**Figure 5.**

**A**

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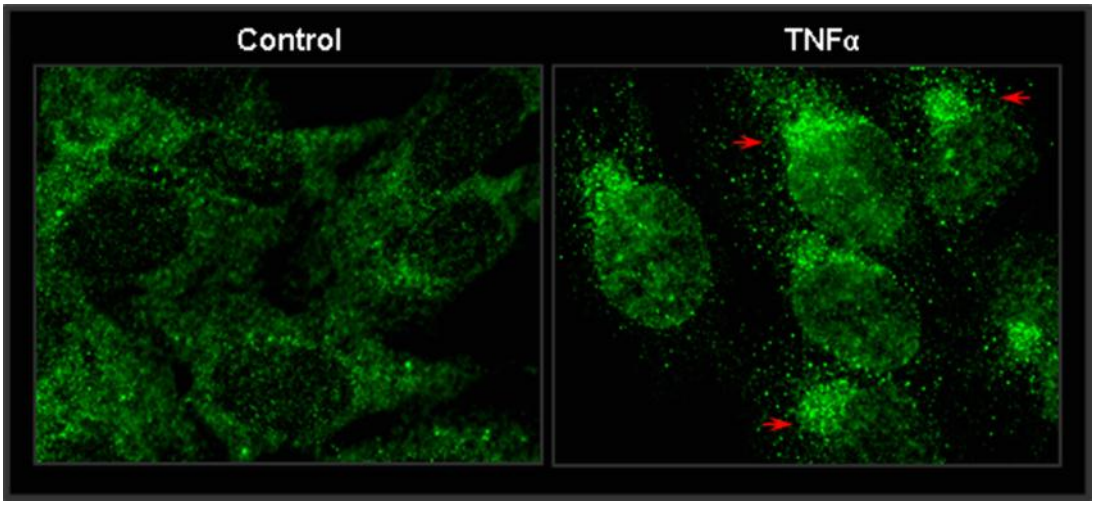


**Figure 5. PKCδ translocates to the nucleus during TNF treatment. (A) Confocal imaging of PKCδ localization. (A )**N27 cells were treated with TNF for 6 h and processed for immunocytochemistry using a rabbit polyclonal PKCδ antibody to the C-terminus of the protein that detects the native and proteolytically cleaved form. An Alexa-488 dye-tagged secondary antibody was used to visualize PKCδ (green) and the nucleus was stained using the TOPRO-3 dye (blue). In untreated controls (top panel), PKCδ was almost completely localized to the cytosol with no evident nuclear localization as seen in the merged images and the XY-plane spatial intensity plots of the green and blue channels. In TNF-treated cells (lower panel), a distinct localization of PKCδ to the nucleus and the peri-nuclear region is readily apparent. **(B)** Inverse grey scale image of PKCδ immunofluorescence staining in TNF-treated cells showing PKCδ accumulation at distinct spatial locations at the perinuclear region (red arrows).

**Figure 6.**

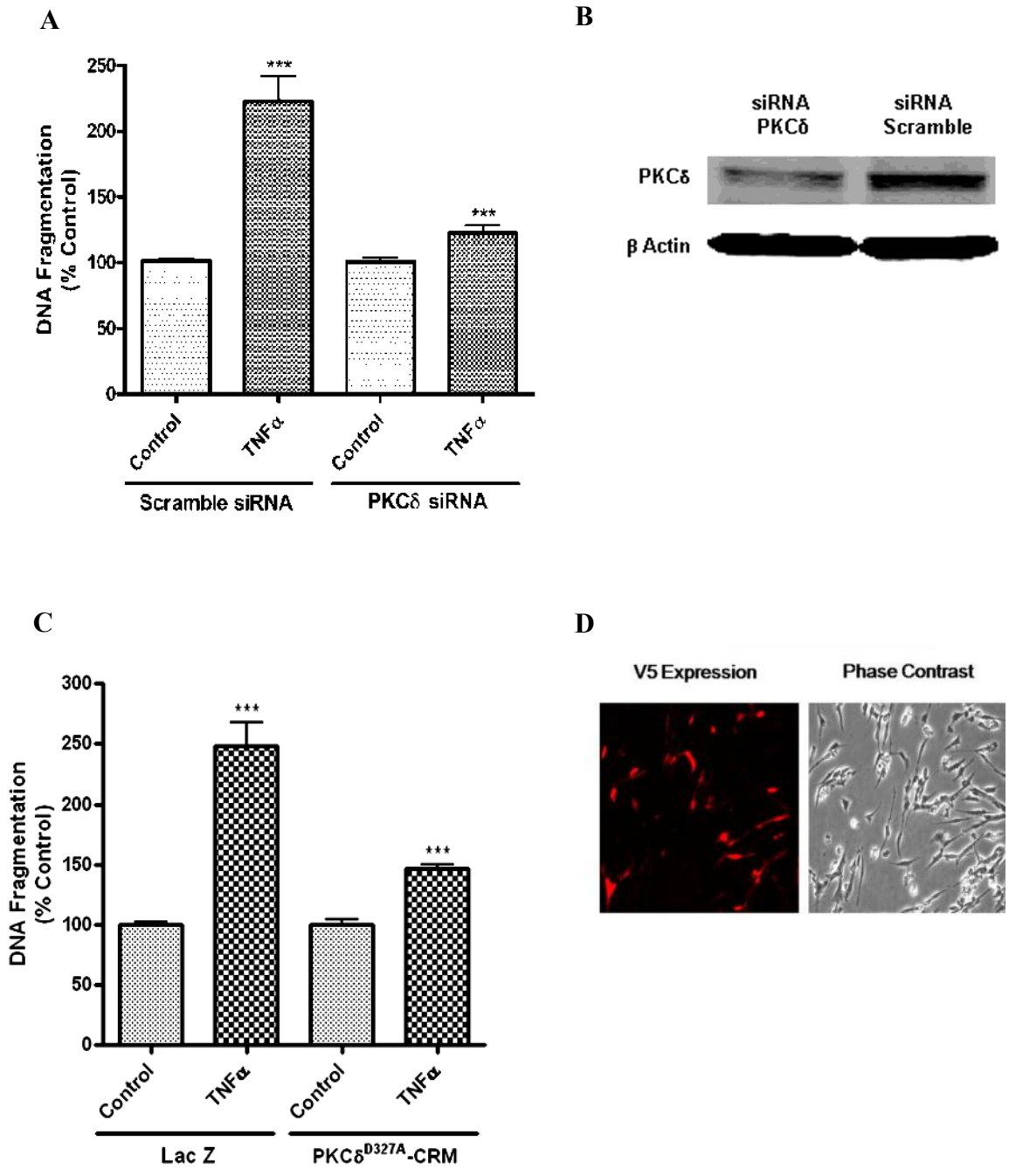


**Figure 6. siRNA knockdown of PKCδ or overexpression of the PKCδ proteolytic cleavage-resistant mutant (PKCδ$^{D327A}$-CRM) protects against TNF toxicity. (A)** DNA fragmentation assay in siRNA transfected cells. N27 cells transfected with either siRNA to PKCδ or scrambled control siRNA were treated with TNF for 16 h and processed for the DNA fragmentation assay to quantify cell death. Suppression of the PKCδ protein level was confirmed by Western blotting **(B)**. TNF-induced DNA fragmentation was significantly reduced in PKCδ siRNA transfected cells confirming the pro-apoptotic role of this kinase. **(C)** DNA fragmentation assay in N27 cells over expressing the cleavage-resistant PKCδ mutant protein (PKCδ$^{D327A}$-CRM). N27 stably expressing the cleavage-resistant PKCδ mutant or the β-galactosidase (LacZ) control gene were treated with TNF for 16 h and processed for the DNA fragmentation assay to quantify cell death. Expression of the mutant PKCδ protein was confirmed by imaging the V5 tag by immunocytochemistry **(D).** DNA fragmentation induced by TNF was reduced in N27 cells expressing the caspase-3 cleavage-resistant mutant protein, indicating the proteolytic cleavage is a necessary event for TNF toxicity in these cells. Data represent the group mean $\pm$ SEM of at least six individual readings. Asterisks (***$p<0.001$) indicate significant differences between TNF-treated groups in scramble siRNA or lacZ expressing cells and PKCδ siRNA or cleavage-resistant mutant expressing cells respectively.

**Figure 7.**

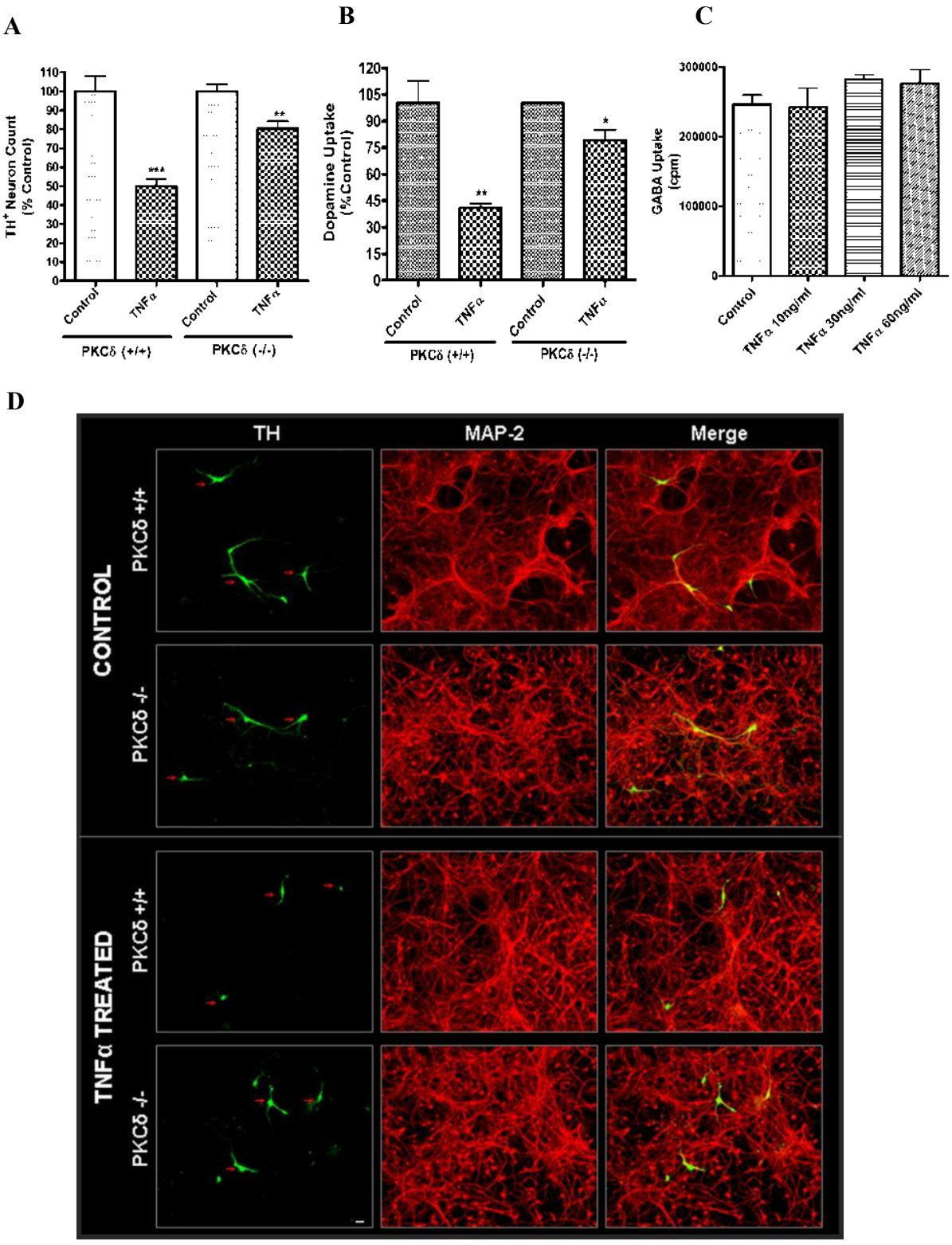
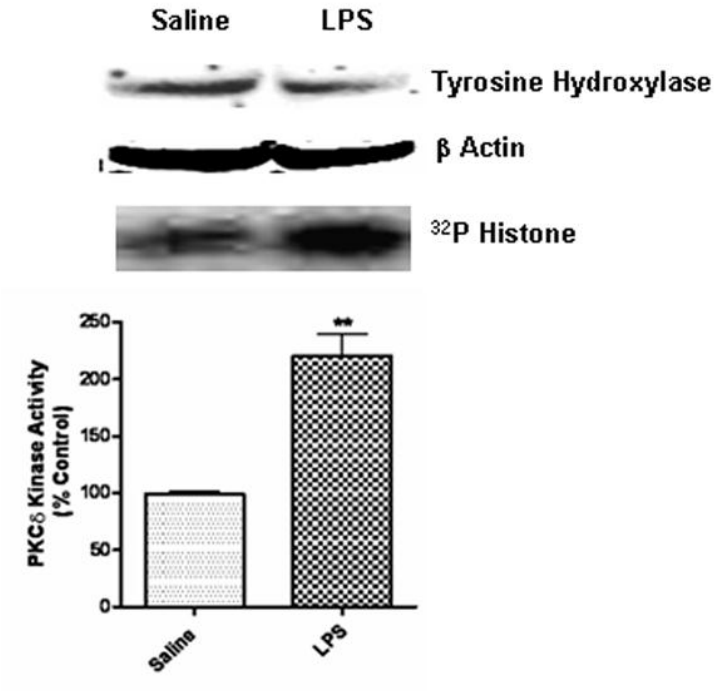


**Figure 7. Primary dopaminergic neurons in EVM cultures from PKCδ knockout mice are protected against TNF toxicity.** Primary mouse EVM neuronal cultures (>95% free of glial cells) were treated with recombinant TNF (30ng/ml) in supplement-free neurobasal medium. TNF was re-added 24 h later for a total treatment time of 48 h. PKCδ wild type (PKCδ +/+) and knockout (PKCδ -/-) EVM cultures were treated in parallel under identical conditions. **(A-C)** TH-positive neuron counts and neurotransmitter uptake assays. Dopaminergic neurons were identified by tyrosine hydroxylase labeling and the number of TH-positive neurons from 8 random fields per well were counted at 20X magnification for each treatment group. Data from 3 independent experiments were combined and expressed as percent control **(A).** The uptake of tritiated [$^3$H] dopamine was determined in TNF-treated cultures from PKCδ WT and knockout mice. The values for non-specific uptake of dopamine obtained in the presence of mazindol were subtracted as background and the data expressed as percent control **(B)**. Combined data from three independent experiments is shown. GABA uptake was also determined in wild type cultures to verify if TNF was toxic to other cell populations under these treatment conditions. **(C)**. Dopamine uptake and TH-positive neuron counts were significantly reduced with TNF treatment in cultures obtained from PKCδ wildtype (+/+) mice while cultures from PKCδ knockout (-/-) mice were resistant to TNF toxicity. No significant changes in GABA uptake were observed. Data represent the group mean $\pm$ SEM of three experiments. Asterisks (***$p<0.001$, **$p<0.01$ and *$p<0.05$) indicate significant differences between control and TNF-treated groups in PKCδ wild type cultures, and also between TNF treatments from PKCδ wildtype (+/+) and PKCδ knockout (-/-) cultures. **(D)** Immunocytochemistry for TH-positive neurons. Dopaminergic neurons were

identified by tyrosine hydroxylase staining (green) and MAP-2 (red) was used as a pan-neuronal marker. Images were acquired at 20X magnification. Representative fields are shown for each treatment. Untreated cultures from both WT and knockout mice (top panel) displayed normal morphology and had similar numbers of dopaminergic neurons. In TNF-treated groups (lower panel), extensive loss of neurites and degeneration of dopaminergic neurons is evident in cultures from PKCδ wildtype (+/+) mice while cultures from PKCδ knockout (-/-) mice have visible branched neurites (red arrows), more numerous cell bodies and are resistant to degeneration induced by TNF, consistent with the established role of this kinase in apoptosis.

**Figure 8.**

**A**

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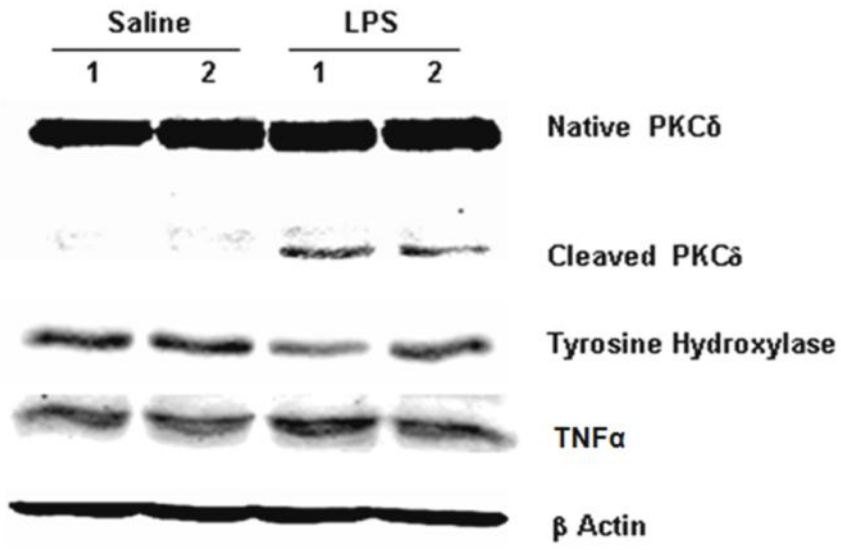

**Figure 8. Proteolytic activation of PKCδ in the mouse substantia nigra during LPS-induced dopaminergic degeneration. (A)** PKCδ kinase activity in mouse substantia nigra tissue. C57BL/6 mice were stereotaxically injected with a single dose of LPS (5 μg) in the SN to elicit dopaminergic degeneration. Mice were sacrificed after 14 days and PKCδ IP-kinase assays were performed on nigral tissue lysates. The kinase activity was quantified by densitometric analysis of the $^{32}$P-Histone bands. Western blots of tyrosine hydroxylase and β-actin were performed on the same lysates to verify accurate dissection of the nigral tissue and protein loading respectively. Significantly higher PKCδ kinase activity was evident in nigral tissues obtained from LPS-injected mice compared to saline controls. Experiments were repeated three times and representative blots are shown (**B)** Proteolytic cleavage of PKCδ in mouse substantia nigra tissue. To determine if the increased PKCδ kinase activity was a result of proteolytic activation, Western blots were performed on nigral tissue lysates obtained from mice injected with either LPS or saline as control. Increased proteolytic cleavage of PKCδ was evident in LPS-injected mice and was essentially absent in the saline-injected group. Blots were also probed for tyrosine hydroxylase and β-actin to confirm accurate dissection of the nigral tissue and equal protein loading. Experiments were repeated 3 times and representative blots are shown. Data represent the group mean $\pm$ SEM. Asterisks (**$p<0.01$) indicate significant differences between saline and LPS-injected mice.

# CHAPTER III

## PROTEIN KINASE Cδ IS INDUCED IN ACTIVATED MICROGLIA AND REGULATES MICROGLIAL NEUROTOXICITY AND DOPAMINERGIC DEGENERATION IN PARKINSON'S DISEASE MODELS

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**Abstract**

Several lines of evidence suggest that chronic microglial activation and neuroinflammation can contribute to the progressive degeneration of dopaminergic neurons initiated by oxidative stress and other neurotoxic mechanisms in Parkinson's disease (PD). In this study we identify that protein kinase Cδ (PKCδ), a member of the novel PKC family, is highly induced in activated microglia and regulates microglial proinflammatory responses. PKCδ mRNA and protein expression is increased in activated primary microglia and BV-2 cells with a concomitant increase in kinase activity. PKCδ showed distinct intracellular localization in activated microglia that appears to be stimulus-specific. Further, primary microglia isolated from PKCδ knockout mice have reduced cytokine and chemokine production, nitric oxide generation and intracellular ROS responses compared to microglia from wild-type mice. PKCδ knockout mice show a dramatic recovery from LPS-induced

systemic inflammation and sickness behavior along with reduced levels of TNFα and other proinflammatory cytokines in the substantia nigra, demonstrating a crucial role for PKCδ in regulating neuroinflammatory responses. In the MPTP model of PD, PKCδ knockout mice had reduced microglial activation and oxidative stress levels in the substantia nigra. Significantly, PKCδ knockout mice showed robust protection against MPTP-induced nigrostriatal dopaminergic degeneration and motor deficits. Taken together, our findings identify PKCδ as a novel regulator of microglial activation and neuroinflammatory processes, and demonstrate that therapeutic targeting of PKCδ could be useful to mitigate progressive nigrostriatal dopaminergic degeneration in PD.

**Introduction**

Parkinson's disease (PD) is the most common neurodegenerative movement disorder affecting around 2% of the population over the age of 60 (Moore et al., 2005; Schapira, 2009). The pathological hallmark of PD is a selective loss of dopaminergic (DA) neurons in the substantia nigra of the midbrain resulting in a depletion of striatal dopamine that is clinically manifest as a range of motor and nonmotor symptoms (Obeso et al., 2010). Sustained microglial activation and neuroinflammation in the SNpc is evident in PD patients and animal models of the disease, being initiated early during dopaminergic degeneration and still apparent in postmortem PD patients (Block et al., 2007; Hirsch and Hunot, 2009; Glass et al., 2010; Tansey and Goldberg, 2010). Compelling evidence from human, animal and epidemiological studies now support a role for neuroinflammatory mechanisms in the pathogenesis and progression of Parkinson's disease with both the innate and adaptive arms

of the immune system being involved. (Wu et al., 2002; Benner et al., 2004; Ghosh et al., 2007a; Brochard et al., 2009; Appel et al., 2010; Tansey and Goldberg, 2010). However, little is known about the process by which microglial cells escape regulatory control and become persistently activated in the disease state and contribute to the slow progressive degeneration of dopaminergic neurons. Microglial NADPH oxidase has been shown to be involved in generation of free radicals in the substantia nigra and can exacerbate dopaminergic degeneration by augmenting local oxidative stress levels (Wu et al., 2002; Wu et al., 2003). Similarly, NFκB activation in microglia has been shown to be a critical regulator of the microglial neurotoxic response by mediating proinflammatory cytokine levels and the regulation of iNOS activity in the substantia nigra (Ghosh et al., 2007a). However the intracellular signaling pathways involved in regulating sustained microglial activation during dopaminergic degeneration remain to be established and could provide potential targets to slow the progression of the disease.

Protein kinase Cδ (PKCδ), a member of the novel PKC isoform family, is highly expressed by dopaminergic neurons in the SNpc, primary neurons and by dopaminergic cell lines (Zhang et al., 2007b). Recent studies from our laboratory and others have shown that PKCδ is proteolytically activated by caspase-3 during dopaminergic cell death and that genetic or pharmacological targeting of PKCδ can protect against dopaminergic degeneration in PD models (Anantharam et al., 2002; Kaul et al., 2003; Zhang et al., 2007a; Hanrott et al., 2008). However, PKCδ has also been shown to regulate inflammatory responses and immune signaling in peripheral immune cells including neutrophils, B cells and macrophages (Tepperman et al., 2000; Bey et al., 2004; Mecklenbrauker et al., 2004; Carpenter and

Alexander, 2008; Kilpatrick et al., 2010). Therefore, we hypothesized that PKCδ could be involved in the microglial activation and thereby regulate the neuroinflammatory response. We tested our hypothesis using microglia isolated from PKCδ knockout mice and in vivo using the systemic LPS model and the classic MPTP mouse model of PD.

Our findings herein identify a novel function for native full-length PKCδ signaling in microglial cells. We demonstrate that PKCδ is highly induced in activated microglia and regulates the microglial proinflammatory response and nigrostriatal dopaminergic degeneration in vivo. Given that critical components of the microglial activation machinery, including NFκB, NADPH and iNOS, are known substrates of PKCδ, our findings have profound implications for targeting the microglial neuroinflammatory response to mitigate progressive nigrostriatal dopaminergic degeneration in PD.

## Materials and methods

### Chemicals and Reagents

DMEM/F-12, RPMI, fetal bovine serum, L-glutamine, IR-dye tagged secondary antibodies, Hoechst nuclear stain, penicillin, and streptomycin and other cell culture reagents were purchased from Invitrogen (Gaithersburg, MD). Recombinant TNFα was purchased from Peprotech (Rocky Hill, NJ), LPS (*E. coli* 0111:B4) was purchased from Sigma and recombinant human alpha synuclein was purchased from rPeptide Inc (Bogart, GA). Aggregation of alpha synuclein was performed according to previously published procedures (Zhang et al., 2005) by incubating the purified recombinant protein at room temperature with agitation for 7 days. The formation of alpha synuclein aggregates was verified by

transmission electron microscopy (Supplementary Figure1). Antibodies for rabbit PKCδ, GRP-78 and NOS2 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse TH antibody was purchased from Chemicon (Temecula, CA) and rabbit Iba1 antibody was from Wako Chemicals (Richmond, VA). $^{32}$P-ATP was purchased from Perkin Elmer (Boston, MA) and the histone substrate from Sigma. The Bradford protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA).

**Animal studies**

The generation and maintenance of PKC delta knockout mice is described in our previous publications (Zhang et al., 2007b). Mice were genotyped as per previously published protocols (Miyamoto et al., 2002). Wild-type C57Bl/6 mice and PKCδ knockout mice were housed under standard conditions of constant temperature (22 ± 1°C), humidity (relative, 30%), and a 12 h light/dark cycle with free access to food and water ad libitum. Six to eight week old mice were used for all studies. The well-characterized acute MPTP mouse model of PD (Wu et al., 2003; Przedborski et al., 2004; Kim et al., 2007; Hu et al., 2008) was primarily used for neuroinflammation and neuroprotection studies. The mice from the MPTP treatment group received 4 i.p. injections of MPTP-HCl (18 mg/kg free base) dissolved in saline at 2-h intervals and control mice received saline injections. The mice were sacrificed 1 to 7 days after the last injection. The nigral neuroinflammatory response was also studied using the systemic LPS injection model (Qin et al., 2007) that has been shown to induce chronic neuroinflammation and progressive dopaminergic degeneration in mice. A single injection of LPS (5 mg/kg, i.p.) was delivered to PKCδ wild-type and knockout mice. Control groups received equivalent injections of saline. Mice were sacrificed 24 to 48 h later.

All animal procedures were approved by the Iowa State University Institutional Animal Care and Use Committee (IACUC).

**Primary microglial cultures and treatments**

Primary microglial cultures were prepared from C57BL/6 postnatal day 1 (P1) mouse pups. The mouse brains were harvested, meninges removed, and then placed in Dulbecco's modified Eagle's medium/F-12 nutrient media (DMEM-F12,GIBCO Cat # 11320) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 U/mL penicillin, 50 μg/mL streptomycin, 2 mM L-glutamine, 100 μM non-essential amino acids, and 2 mM sodium pyruvate (Invitrogen). The brain tissue was then incubated in 0.25% trypsin for 30 min with gentle agitation. The trypsin reaction was stopped by adding an equal volume of DMEM/F12 complete medium and the brain tissue was washed 3 times. The tissue was then triturated gently to prepare a single cell suspension and then passed through a 70 μm nylon mesh cell strainer to remove tissue debris and aggregates. The cell suspension then was made up in DMEM/F12 complete medium and seeded into T-75 flasks. The cells were placed in humidified $CO_2$ incubator at 37°C, the medium was changed after 4 to 5 days and the mixed glial cells were grown to confluence. Microglial cells were separated from confluent mixed glial cultures by differential adherence and magnetic separation to >97% purity as described in our recent publication (Gordon et al., 2011). Microglia were allowed to recover for at least 48 hours after plating. Primary microglia were treated in DMEM/F12 complete medium containing 2% FBS.

**Lentiviral transduction of primary microglia and shRNA knockdown of PKCδ**

Lentiviral transduction and knockdown of PKCδ in primary microglia was performed according to previously published reports (Takahashi et al., 2005) with some modifications. Pre-validated lentiviral particles expressing PKCδ-specific shRNA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) (catalog # sc-36246-V) along with non-specific control shRNA lentiviral particles (catalog # sc-108080). Titers of the viral particles were around $10^6$ multiplicity of infection. Purified microglial cells were seeded at 3 x $10^5$ cells/ml into 24-well plates and allowed to attach overnight. The next day, lentiviral particles and 5 μg/ml of polybrene (Millipore Inc.) were added to the culture and centrifuged for 60 min at 30°C. The plates were further incubated for 3 h at 37°C before removing the supernatant containing the viral particles and replacing with complete DMEM/F12 medium containing 10% FBS. The efficiency of transduction was around 90% as determined by GFP expression 48 h later and all treatments were performed at this time point. Lentiviral shRNA knockdown of the PKCδ protein in primary microglia was confirmed by Western blotting using a rabbit polyclonal antibody.

**Immunohistochemistry and Immunofluorescence studies**

Mice were deeply anesthetized with ketamine/xylazine mixture and then perfused transcardially with freshly prepared 4% PFA. Brains were removed, postfixed in PFA for 48 h and 20 micron sections were cut using a freezing microtome (Leica Microsystems). Section from the substantia nigra and other regions of interest were processed for immunohistochemistry. Antigen retrieval was performed by heating the free-floating sections in citrate buffer (10mM sodium citrate, pH 8.5) for 30 minutes at 90°C. Sections were then

washed several times in PBS and blocked with PBS containing 2% BSA, 0.2% TritonX-100 and 0.05% tween-20 for 2 h at room temperature. Sections were then incubated with antibodies directed against PKCδ (rabbit polyclonal, 1:500) and TH (mouse monoclonal, 1:2000) overnight at 4°C and washed several times in PBS. The sections were incubated with Alexa dye-conjugated secondary antibodies for 1 h at room temperature and added then stained with Hoechst dye. Sections were mounted on slides using prolong antifade gold mounting medium (Invitrogen) according to the manufacturer's instructions. Samples were visualized using a Nikon inverted fluorescence microscope (TE-2000U) and images were captured using a spot digital camera (Diagnostic Instruments Inc, Sterling Heights, MI) PKCδ immunofluorescence in primary microglia was performed according to previously published protocols with some modifications. Briefly, microglial cells were grown on poly-D-lysine coated coverslips and treated 48 h later. At the end of treatments, cells were fixed with 4% paraformaldehyde, washed in PBS and incubated in blocking buffer (PBS containing 2% BSA, 0.5% TritonX and 0.05% Tween) for 1 h at room temperature. The coverslips were then incubated overnight at 4°C with respective primary antibodies diluted in PBS containing 2% BSA. Samples were then washed several times in PBS and incubated with Alexa dye-conjugated secondary antibodies. The nucleus was labeled with Hoechst stain (10 μg/ml) and coverslips were mounted with Fluoromount medium (Sigma Aldrich) on glass slides for visualization.

**qRT-PCR for gene expression analysis**

RNA isolation from primary microglial cells and brain tissue samples was performed using the Absolutely RNA Miniprep Kit and 1 μg of total RNA was used for reverse

transcription with the AffinityScript qPCR cDNA synthesis system (Agilent Technologies) according to the manufacturer's instructions. Quantitative SYBR Green real-time PCR assays for PKCδ and proinflammatory cytokine gene expression analysis was performed using the RT² SYBR Green Master Mix with prevalidated qPCR primers (SA biosciences qPCR assay system) Catalog numbers of the primers were PKCδ - PPM03810E, IL1β - PPM03109E, IL12 - PPM03020E, TNFα - PPM03113F and iNOS - PPM02928B. The mouse 18SrRNA gene (catalog number - PPM57735E) was used as the housekeeping gene for normalization. For each primer, the amount of template which provided the maximum efficiency without inhibition of the PCR reaction was determined during initial optimization experiments. For all experiments, dissociation curves were run according to the manufacturer's instructions and melting curve analysis was performed to ensure a single peak was obtained at the right melting temperature without non-specific amplicons. The fold change in gene expression was determined by the $\Delta\Delta C_t$ method using the threshold cycle ($C_t$) value for the housekeeping gene and for the respective target gene of interest for each sample.

**Western blotting**

Brain tissue and microglial cell lysates were prepared using a modified RIPA buffer and normalized for equal amounts of protein using the BCA protein assay kit (Pierce Biotechnology). Equal amounts of protein (30 to 50 µg) were loaded for each sample and separated on either 12% or 15% SDS-PAGE gels depending on the molecular weight of the target protein. After separation, proteins were transferred to a nitrocellulose membrane and the nonspecific binding sites were blocked for 1 h using a blocking buffer specifically formulated for fluorescent Western blotting (Rockland Immunochemicals) and then probed

with the respective primary antibodies overnight at 4°C. After incubation the membranes were washed 3 times with PBS containing 0.05% Tween and IR-dye tagged secondary antibodies (1:5000 Molecular Probes) were added and the membranes visualized on the Odyssey infrared imaging system. Primary antibodies used were rabbit polyclonal PKCδ (1:500), mouse monoclonal TH (1:2000), rabbit polyclonal iba1 (1:200), mouse monoclonal gp91 PHOX (1:2000) and rabbit iNOS (1:250). Beta Actin and Tubulin (1:5000) were used as loading controls.

**PKCδ immunoprecipitation-kinase assays**

PKCδ IP kinase activity assays were performed as described previously (Anantharam et al., 2002; Kaul et al., 2005; Zhang et al., 2007a) with some modifications. Briefly, primary microglia or BV-2 cells were collected after treatments, washed in ice cold PBS and resuspended in a mild RIPA lysis buffer containing protease and phosphatase inhibitor cocktail (Pierce Biotechnology).The lysates were then centrifuged at 13,000 rpm for 45 min. The supernatant was collected and protein concentration determined using the Bradford assay (Bradford, 1976). Protein concentration was normalized and 500 µg of total protein in a 250 µl reaction volume was immunoprecipitated overnight at 4°C using 5 µg of PKCδ antibody. The next day, protein-A agarose beads (Sigma-Aldrich) were added and the samples incubated for 1 h at room temperature. The protein A-bound antibody complexes were collected and washed 3 times in 2X kinase assay buffer (40 mM Tris, pH 7.4, 20 mM $MgCl_2$, 20 µM ATP, and 2.5 mM $CaCl_2$), and then resuspended in the same buffer. The kinase reaction was started by adding 40 µl of the reaction buffer containing 0.4 mg of histone H1, 50 µg/ml phosphatidylserine, 4 µM dioleoylglycerol, and 10 µCi of [$\gamma$-$^{32}$P] ATP

at 3000 Ci/mM to the immunoprecipitated samples. The samples were then incubated for 10 min at 30°C and the kinase reaction stopped by adding 2X SDS loading buffer and boiling for 5 min. Proteins were separated on a 15% SDS-PAGE gel and phosphorylated histone bands were imaged on a Fujifilm FLA 5000 imager. Image analysis and band quantification was performed using the Fujifilm Multigauge software package.

**Microglial nitric oxide detection**

Nitric oxide production by primary microglia was measured indirectly by quantification of nitrite in the supernatant using the Griess reagent (Sigma Aldrich). Magnetically separated microglia were plated in poly-D-lysine coated 96-well plates at $10^5$ cells per well. Cells were treated with 100 ng/mL LPS for 24 h and 100 µL of supernatant was collected from each well and an equal volume of the Griess reagent was added. The samples were incubated on a plate shaker at room temperature for 15 min until a stable color was obtained. The absorbance at 540 nm was measured using a Synergy 2 multi-mode microplate reader (BioTek Instruments) and the nitrite concentration determined from a sodium nitrite standard curve.

**Multiplex cytokine and chemokine Luminex immunoassays**

Primary microglia were obtained from PKCδ wild-type and knockout mice on the same day and seeded in poly-D-lysine coated 96-well plates at 0.5 x $10^5$ cells per well. The cells were treated for 24 h with 100 ng/mL LPS, 30 ng/ml TNF or 300 nM aggregated alpha synuclein. After treatment, 50 µL of supernatant from each well was collected and frozen at -80°C. The levels of cytokines and chemokines in the supernatants were determined using the Luminex bead-based immunoassay platform (Vignali, 2000) using pre-validated multiplex

kits (Milliplex mouse cytokine and chemokine panels – Millipore Corporation) according to the manufacturer's instructions

**Intracellular reactive oxygen species (iROS) detection**

Intracellular reactive oxygen species (iROS) were determined using the fluorescent probe 2',7'-dichlorodi-hydrofluorescein diacetate (DCFH-DA) (Calbiochem), according to previously published reports (Zhang et al., 2005; Qian et al., 2007). Magnetically separated microglia from PKCδ wild-type and knockout mice were plated at $10^5$ cells per well in 96-well plates and allowed to attach overnight. Before treatment, cells were washed in HBSS and incubated with 40 μM DCFH-DA in HBSS containing 2% FBS for 30 min. Cells were then treated with 100 ng/mL LPS, 30 ng/mL of recombinant mouse TNF alpha or 300 nM aggregated alpha synuclein. for 6 h. The fluorescence intensity was determined using a Synergy-2 multi-mode microplate reader at an excitation of 485/20 nm and an emission of 530/25 nm. The fluorescence value from the control cultures was subtracted as background and the increase in fluorescence with treatments was expressed as increased iROS as previously described (Zhang et al., 2005; Qian et al., 2007).

**Tyrosine hydroxylase (TH) immunostaining and stereological counting of TH positive neurons**

TH- diaminobenzidine (DAB) immunostaining was performed on striatal and substantia nigral sections as described previously (Ghosh et al., 2010). Briefly, PKCδ wild-type and knockout mice were injected with MPTP or saline and sacrificed 7 days after treatment. The mice were perfused with 4% paraformaldehyde (PFA) and brains were post-fixed with PFA for 48 h before storage in 30% sucrose. Frozen blocks were prepared from

the fixed brains and were cut using a cryostat into 30 µm coronal sections. Sections were probes with an anti-TH antibody (Calbiochem; rabbit anti-mouse, 1:1600) overnight at 4°C and then incubated with biotinylated anti-rabbit secondary antibody. The sections were then treated with Avidin peroxidase (Vectastatin ABC Elite kit). The DAB reagent provided with the kit was used for producing the brown colored stain. The total number of TH-positive neurons in SNpc was counted by unbiased stereology using the Stereo Investigator software (MicroBrightField, Inc., Williston, VT) and the optical fractionator method as described in our previous publications (Ghosh et al., 2007b; Ghosh et al., 2010)

**HPLC analysis of striatal dopamine and its metabolite levels**

The amount of striatal dopamine (DA) and its metabolites (DOPAC and HVA) was quantified by high-performance liquid chromatography (HPLC) with electrochemical detection as described previously (Zhang et al., 2007 and Ghosh et al., 2010). Briefly, 7 days after the last MPTP injection, PKCδ wild-type and knockout mice were sacrificed and striata were collected, weighed and stored at -80°C. Neurotransmitters from striatal tissues were extracted in a 0.1 M perchloric acid solution containing 0.05% $Na_2EDTA$ and 0.1% $Na_2S_2O_5$) and isoproterenol (used as an internal standard). Extracts were filtered in 0.22 µm spin tubes, and 20 µl of the sample was loaded for analysis. Dopamine, DOPAC and HVA were separated isocratically in a reversed-phase column using a flow rate of 0.7 ml/min. An HPLC system (ESA Inc., Bedford, MA, USA) with an automatic sampler equipped with a refrigerated temperature control (model 542; ESA Inc.) was used for all experiments. The electrochemical detection system consisted of a Coulochem model 5100A with a microanalysis cell (model 5014A) and a guard cell (model 5020) (ESA Inc.). Standard

catecholamine stock solutions of 1 mg/ml were prepared in perchloric acid solution and further diluted to a final working concentration of 50 pg/µl prior to injection. All data acquisition and analysis were performed using the EZStart HPLC Software (ESA Inc.).

**Behavioral studies and locomotor activity measurements**

Five days after MPTP treatment, wild-type and knockout mice were tested for behavioral parameters and locomotor activity. An automated instrument (AccuScan, model RXYZCM-16, Columbus, OH, USA) was used to monitor the locomotor activity of mice. The activity chamber was 40×40×30.5 cm in dimensions, made of clear Plexiglas and covered with a Plexiglas lid with ventilation holes. Infrared monitoring sensors were located every 2.54 cm along the perimeter (16 infrared beams along each side) and 2.5 cm above the floor. Two additional sets of 16 sensors were located 8.0 cm above the floor on opposite sides. Prior to any behavioral studies, mice were placed inside the infrared monitor for 5 min daily for 3 consecutive days to train them. The various locomotor activity parameters were determined simultaneously over a 10 min testing session and were presented as horizontal movement, vertical movement, total distance travelled (cm), total movement time, and rearing activity. Data was collected and analyzed by a VersaMax Analyzer (AccuScan, model CDA-8, Columbus, OH). For Rotarod performance analysis, a 20 rpm speed setting was used to monitor the foot movement of mice as described in our previous publication (Ghosh et al., 2010). A total of 6 trials per mouse was performed and mice were given a 7-10 min rest interval between trials.

**Data analysis**

Data analysis was performed using the Prism 4.0 software package (GraphPad Software, San Diego, CA). The data was first analyzed using one-way ANOVA and then Bonferroni's post-test was performed to compare all treatment groups. Differences of $p<0.05$ were considered statistically significant. The Student's t-test was used when differences between two groups were being compared.

**Results**

**Protein Kinase Cδ is highly induced during microglial activation**

Sustained nigral neuroinflammation and reactive microgliosis are evident in Parkinson's disease (PD) patients and all animal models of the disease and believed to contribute to disease pathogenesis by driving progressive dopaminergic neuron loss. Previous studies from our laboratory and other independent research groups have shown that proteolytic activation of PKCδ occurs in dopaminergic neurons and that therapeutic targeting of PKCδ can provide neuroprotection in the mouse MPTP model of PD (Anantharam et al., 2002; Kanthasamy et al., 2003; Kaul et al., 2003; Zhang et al., 2007a; Hanrott et al., 2008; Sun et al., 2008; Cunningham et al., 2009). Since reactive microgliosis is apparent in PD patients and animal models of the disease, we tested if PKCδ could play a role in microglial activation. Using an antibody directed against the C-terminus of the protein we initially determined if PKCδ proteolytic activation occurs in primary microglia and BV-2 cells using various activation stimuli relevant to PD. Surprisingly, we did not detect proteolytic cleavage of PKCδ during microglial activation in any of the treatments. Instead,

to our surprise, we found that the native full length PKCδ protein is highly induced with sustained microglial activation (Fig.1). In primary microglia, the full length PKCδ protein was upregulated following microglial activation with LPS and aggregated alpha synuclein (Fig. 1A). In BV-2 microglial cells, a commonly used microglial cell line, we observed significant induction of PKCδ following stimulation with LPS and TNFα. Further, active MMP-3, a protease released from degenerating dopaminergic neurons which activates microglia (Kim et al., 2005; Kim et al., 2007), also induced significant induction of PKCδ protein levels in BV-2 cells (Fig. 1C). To validate our Western blot results for PKCδ induction at the gene level, we did qRT-PCR experiments for changes in PKCδ gene expression levels in primary microglia stimulated with LPS, TNF and aggregated alpha synuclein for 24 h. We observed significant increases (threefold to sixfold) in PKCδ gene expression in activated microglia. In primary microglial cells, TNFα caused the highest increase in PKCδ mRNA levels followed by LPS and aggregated alpha synuclein. Together, these results demonstrate for the first time that the PKCδ gene and protein levels are both increased in activated microglial cells. To validate our preliminary findings from primary microglia in vivo, we used two well characterized mouse models relevant to neuroinflammation and PD: the classic MPTP model (Czlonkowska et al., 1996; Du et al., 2001; Wu et al., 2003; Przedborski et al., 2004) and the more recent single dose systemic LPS model (Cunningham et al., 2005; Qin et al., 2007). In both the acute MPTP model and the systemic LPS model, a strong microglial activation response is observed 24 to 48 h after treatment (Wu et al., 2003; Qin et al., 2007) in the mouse substantia nigra. Therefore, we determined the changes in PKCδ gene expression levels by qRT-PCR at the 24 h time point

in nigral tissue obtained from MPTP and LPS-injected mice. In agreement with our in vitro results we observed around a ninefold increase with the MPTP model and around fivefold increase with LPS treatment in PKCδ mRNA levels in the mouse substantia nigra (Fig. 1E). Collectively these results demonstrate that PKCδ is induced during microglial activation and could play an important role in regulating the microglial neuroinflammatory response.

**PKCδ kinase activity is increased in activated microglia**

Having confirmed that the full length PKCδ protein was being induced during sustained microglial activation, we determined if there was a concomitant increase in PKCδ kinase activity. Since PKCδ is member of the novel PKC family (PKCs :δ, ε,η, θ) it does not require calcium for activation, although lipid cofactors such as DAG are necessary for maximum kinase activity. Further, we did not detect a proteolytically cleaved PKCδ fragment in activated microglia suggesting that classical full length PKC activation mechanisms involving multiple priming phosphorylations at the activation loop may be involved (Steinberg, 2004). We used PKCδ immunoprecipitation-kinase assays with a histone substrate and $^{32}$P-ATP to measure the kinase activity of PKCδ in primary microglia and BV-2 cells activated with LPS, TNFα and aggregated alpha synuclein. In unstimulated primary microglial cells we observed only low levels of basal PKCδ kinase activity, however upon stimulation with LPS, TNFα or aggregated alpha synuclein there was a sharp increase in PKCδ kinase activity (Fig. 2A). The increase in PKCδ kinase activity followed a similar trend as the mRNA levels (Fig. 1D) with TNF having the highest levels of PKCδ kinase activity followed by LPS and aggregated alpha synuclein. The recombinant PKCδ protein was used as a positive control. We obtained similar results with BV-2 microglial cells under

the same treatment conditions (Fig. 2B). Together these results demonstrate that the PKCδ protein is induced during microglial activation along with a concomitant increase in its kinase activity. In contrast to the proteolytic activation mechanism of PKCδ by caspase-3 cleavage during dopaminergic cell death, it appears that in microglia, PKCδ activation is likely to occur through a series of priming phosphorylations at its activation loop, turn motif and hydrophobic motif as reported in other immune cells. The specific mechanisms of PKCδ activation in microglia and its potential substrates are currently under investigation in our laboratory. Interestingly, the induction and activation of PKCδ in primary microglia by diverse activation stimuli such as LPS, TNFα and aggregated alpha synuclein suggests that PKCδ signaling could be a common pathway regulating microglial activation.

**PKCδ localization in activated primary microglia**

Having confirmed that PKCδ is both induced and activated in cultured microglia we studied the subcellular localization of this kinase during microglial activation to better understand its function and identify potential substrates. Several reports have characterized the dynamic spatial and temporal distribution of PKCδ in various cell types and shown that the subcellular localization of PKCδ determines its survival or apoptotic functions (Mecklenbrauker et al., 2004; Gomel et al., 2007; Humphries et al., 2008). In cardiomyocytes for example, native PKCδ translocates from the nucleus to the perinuclear region and to the cytoskeleton. Nuclear and mitochondrial localization of PKCδ is well established during proapoptotic signaling in most cell types. On the other hand, membrane translocation and nuclear shuttling of PKCδ has been reported during immune cell activation. While the expression of various PKC isoforms has been reported previously in microglia and

astrocytes, the localization dynamics of PKCδ during microglial activation has not been determined. We used immunocytochemistry to determine the subcellular localization of PKCδ in primary microglia stimulated with LPS, TNFα and aggregated alpha synuclein. The chaperone protein GRP-78 was used to label the ER, and the nucleus was labeled with Hoechst. In unstimulated primary microglia, PKCδ was largely localized to the cytosol with some diffuse staining evident in the nucleus. Stimulation of primary microglia with LPS caused an intense and dramatic localization of PKCδ to the nucleus and the endoplasmic reticulum network (Fig.3) as seen by colocalization with both Hoechst and GRP-78. When compared to other treatments, LPS also induced the most dynamic change in microglial morphology with increased hypertrophy and processes. TNF alpha treated microglia showed distinct morphology with increased spiny processes but PKCδ remained largely in the cytosol with only diffuse staining in the nucleus similar to controls. The increased PKCδ expression level with TNF treatment was also evident as brighter PKCδ fluorescence in TNF treated cells. In primary microglia treated with aggregated alpha synuclein, PKCδ was largely localized to the cytoplasm, and a distinct exclusion of PKCδ from the nucleus was evident as seen by almost no colocalization with the Hoechst stain. Again, increased PKCδ expression was apparent as brighter PKCδ fluorescence staining in alpha synuclein-treated cells. The dynamic localization of PKCδ in primary microglia activated by distinct pathways indicates that PKCδ could have different substrates and subcellular functions in each case. PKCδ has been shown to act in the NFkB pathway possibly as an IkB kinase in other cell types (Rahman et al., 2001; Minhajuddin et al., 2009). The NADPH oxidase system is another relevant target for PKCδ phosphorylation in these cells and has been shown to be critical for

microglial neurotoxicity and oxidative stress in PD models (Wu et al., 2002; Gao et al., 2003; Wu et al., 2003). The p47 PHOX subunit of the NADPH oxidase complex in particular is a bona fide substrate for PKCδ and could be a common target for regulation of the microglial activation response by diverse stimuli.

**Knockdown of PKCδ by shRNA attenuates LPS-induced nitric oxide production and pro-inflammatory cytokine release**

Since PKCδ was highly induced during microglial activation along with a concomitant increase in its kinase activity, we reasoned that the suppression of PKCδ protein levels could be used to determine its role in regulating the microglial response. We used a lentiviral shRNA system to specifically suppress PKCδ protein levels in primary microglia cells and studied the microglial activation response with LPS stimulation. Successful lentiviral infection of primary microglia was confirmed by the visualization of green fluorescent protein co-expressed with the shRNA (Fig. 4A). In pilot experiments we obtained optimal shRNA suppression of PKCδ protein levels around 72 h after lentiviral infection as determined by Western blotting (Fig. 4B) and primary microglia were treated at this time point for 24 h with 100 ng/ml of LPS. LPS-induced nitric oxide production was significantly reduced ($p < 0.001$) in primary microglia transfected with PKCδ shRNA (Fig. 4C). We also determined the levels of typical proinflammatory cytokines released from primary microglia as a measure of the activation response. In microglia transfected with PKCδ shRNA, the LPS-induced release of IL-6 and TNFα were both significantly reduced compared to microglia transfected with non-specific shRNA (Fig.4D). Together these results suggest that PKCδ can regulate the proinflammatory responses of microglia induced by LPS.

Interestingly, in the same supernatants the levels of LPS-induced IL-12 were not significantly different between in PKCδ shRNA transfected microglia suggesting PKCδ can selectively regulate specific cytokine pathways during LPS-stimulation.

**Microglia from PKCδ knockout mice have reduced cytokine and chemokine production**

To establish the role of PKCδ in regulating the microglial activation response, we used primary microglia isolated from PKCδ knockout (-/-) mice. Primary microglia obtained from wild-type and knockout mice were separated on the same day from mixed glial cultures, seeded at equal density and treated in parallel with LPS, TNF and aggregated alpha synuclein for 24 h. The levels of various cytokine and chemokines in the supernatant were determined using a multiplex bead-based Luminex assay system (Millipore Bioplex). In LPS-treated microglial cells, the levels of several cytokines were significantly reduced in microglia from PKCδ knockout (PKCδ -/-) mice including IL-6, IL-1α and IL-12 p70 (Fig. 5A). The production of several chemokines was also reduced in PKCδ knockout mouse microglia including MIP1α, KC (CXCL-1), MIP-2, IP-10, KC and LIX (Fig. 5B). The levels of several other cytokines and chemokines in the same supernatant remained unchanged between wild-type and PKCδ knockout microglia suggesting a selective regulation of specific cytokine and chemokine pathways by PKCδ. We also measured cytokine responses in primary microglia treated with TNFα and aggregated alpha synuclein. In TNFα treated primary microglia, the levels of IL-6, IP-10, KC, RANTES, MCP-1, MIP-2 and g-CSF were significantly reduced (Fig. 6A) . Interestingly, the levels of MIP1α and MIP1β were increased in PKCδ knockout mouse microglia in contrast to LPS-treatment where MIPα was reduced and MIP1β did not change significantly. We presume this is likely due to the different signaling pathways by

which LPS and TNF activate microglial cells (TLR-4 versus TNF receptors), and a differential involvement of PKCδ in these pathways as evidenced by the distinct spatial localization of PKCδ following LPS and TNF treatments (Fig. 3B). In primary microglia treated with low dose aggregated alpha synuclein, we did not observe major changes with cytokine production similar to previous reports (Zhang et al., 2005). TNFα, MCP-1 and IP-10 were slightly elevated with alpha synuclein treatment in microglia from wild-type mice and were significantly reduced in microglia from the PKCδ knockout mice (Fig. 6C). Other cytokines did not increase appreciably with alpha synuclein treatment. Collectively, these results point to a profound and selective regulation of microglial cytokine and chemokine regulation by PKCδ signaling. These results also support a role for PKCδ as a common downstream kinase regulating microglial activation by different distinct stimuli

**Microglia from PKCδ knockout mice have reduced nitric oxide production and intracellular ROS generation**

Nitric oxide production and intracellular ROS (iROS) generation are important aspects of the microglial activation response and are particularly relevant in PD models where deregulated microglial activation is believed to exacerbate dopaminergic degeneration by augmenting oxidative stress (Block et al., 2007). We compared the nitric oxide production and intracellular ROS generation in microglia isolated from wild-type and PKCδ knockout mice. Nitric oxide production was measured indirectly using the Griess assay. LPS treatment for 24 h induced a sharp increase in nitrite levels (~ 35 μM) in wild-type (PKCδ +/+) microglia, while in microglia from knockout mice (PKCδ -/-) there was only a modest increase in nitrite levels (~ 10 μM) with LPS treatment. Significant differences ($p < 0.001$)

were obtained between LPS-treated microglia from wild-type and PKCδ knockout mice (Fig. 7A). Both TNF (30 ng/ml) and aggregated alpha synuclein (300 nM) did not increase basal levels of nitric oxide production in primary microglia up to 48 h compared to untreated controls (data not shown). The original study by Zhang et al. (2005) using aggregated alpha synuclein did not report significant increases in nitrite production even at the highest doses tested. We also tested the intracellular ROS generation response in primary microglia obtained from PKCδ wild-type and knockout mice. Both LPS and TNFα increased intracellular ROS levels in primary microglia from wild-type mice at 6 h,, whereas primary microglia from PKCδ knockout mice that were seeded at the same density and treated in parallel had significantly lower levels ($p < 0.01$) of intracellular ROS (Fig. 7B,C). Together, these results further support an important role for PKCδ signaling in the regulation of different aspects of the microglial proinflammatory response beyond cytokine production. Based on its known substrates in immune cells it is possible that PKCδ could be a key upstream component of important immune-regulatory signaling pathways such as NFκB or the NADPH oxidase complex, both of which are known to be substrates for PKCδ phosphorylation in other cell types. The iNOS enzyme is also a known target for PKCδ regulation (Ginnan et al., 2006) either directly or via the NFκB signaling pathway.

**PKCδ knockout mice are resistant to LPS-induced sickness behavior and motor deficits**

To validate our results obtained with isolated primary microglia in animal models of PD, we used both the systemic LPS model (Qin et al., 2007) and the well established MPTP model of dopaminergic degeneration (Wu et al., 2003; Przedborski et al., 2004). In the LPS model of PD, a single dose of LPS-injected systemically in mice caused sustained

neuroinflammation in the brain and progressive dopaminergic degeneration almost a year later. Other groups have also used multiple dosing regimens for LPS to induce dopaminergic degeneration at earlier time points (Lee et al., 2008). A wealth of evidence now implicates a role for chronic neuroinflammation in the progression of PD and more recently that systemic inflammation can be an important factor as well. We injected PKCδ wild-type (+/+) and PKCδ knockout mice (-/-) with a single dose of LPS (5 mg/kg) and compared the behavioral and neuroinflammatory responses. To our surprise, the PKCδ knockout mice injected with LPS showed a dramatic recovery from endotoxic shock. While the wild-type mice showed typical signs of endotoxic shock at around 48 h with hunched posture and minimal movement, the PKCδ knockout mice started to recover at around 24 h after injection and had almost normal behavioral responses at 48 h. To confirm these surprising observations, we performed extensive behavioral and locomotor analyses on LPS-injected mice. Age-matched PKCδ wild-type and knockout mice were injected with a single dose of 5 mg/kg LPS (ip) and behavioral activity was assessed using the VersaMax Analyzer and by rotarod performance tests 48 hours later. As shown in the versaplot of locomotor activity (Fig. 8A), LPS-injected wild-type (PKCδ +/+) mice exhibited very little movement and overall locomotor activity while the knockout mice (PKCδ -/-) had almost normal behavioral responses and locomotor activity. We also quantified the horizontal and vertical activity in these mice using the VersaMax Analyzer and obtained the same results. The recovery of the PKCδ knockout mice from endotoxic shock was also readily apparent in the classic rotarod performance test (Fig. 8D). While LPS-injected wild-type mice spent almost no time on the rotarod, the LPS-injected PKCδ knockout mice spent almost as much time as the saline-injected group.

Although the cellular and molecular mechanisms for this phenomenon are not immediately apparent, these dramatic results support a crucial role for PKCδ in regulating the systemic inflammatory response to LPS. Studies are currently ongoing in our laboratory to investigate the potential mechanisms involved.

**PKCδ knockout mice have reduced nigral proinflammatory responses in the following LPS-induced neuroinflammation**

After our behavioral studies, which demonstrated that PKCδ knockout mice were resistant to LPS-induced endotoxic shock and systemic inflammation, we tested the neuroinflammatory responses of these mice in relevance to PD by studying the proinflammatory cytokine responses and microglial activation status in the substantia nigra of these mice. In the original study by Qin et al. (2007), the authors found that the levels of TNFα were chronically elevated in the brain and that the TNF signaling pathway was responsible for transfer of the systemic inflammation into the brain (Qin et al., 2007). Using qRT-PCR we determined the changes in gene expression levels of the proinflammatory cytokines TNFα, IL1β and IL-12 in substantia nigra tissue of LPS-injected PKCδ wild-type and knockout mice. The gene expression studies were performed 48 h after the LPS injection, the time point by which the PKCδ knockout mice had recovered from LPS-induced sickness behavior. Consistent with the results from the previous report with this model we observed highly elevated levels of TNFα mRNA in (20- to 60-fold over saline controls) in nigral tissue obtained from wild-type C57 black mice (Fig. 8A). However the PKCδ knockout mice had only about a threefold increase in TNFα mRNA at 48 h over the saline-injected control group. We obtained similar results with IL-12 as well. IL-1β expression was only slightly

increased in LPS-injected wild-type mice but the fold change was significantly less in LPS-injected knockout mice (Fig. 8A). The almost normal levels of TNFα in the PKCδ knockout mice is particularly relevant based on the studies of Qin et al (2007) with this model where sustained elevated TNF levels were detected for up to 10 months after the single LPS injection. A wealth of other independent studies also supports a pathogenic role for TNF in the degenerative process in both inflammatory and neurotoxic models of PD. In particular the neuroprotection demonstrated with dominant negative TNF in animal models of PD suggests that this pleotropic cytokine could be crucial to the degenerative process in the substantia nigra by neuroinflammatory mechanisms. (Sriram et al., 2002; Ferger et al., 2004; McCoy et al., 2006; Sriram et al., 2006; McCoy et al., 2008). Our data here showing that TNF levels are significantly reduced in PKCδ knockout mice injected with LPS suggests that PKCδ could be regulating TNFα production possibly through the NFκB pathway. We also tested the levels of Iba1 and iNOS as markers of the neuroinflammatory response in the SN tissue following LPS injection. Both iNOS and Iba1 were increased at 48 h post LPS injection in PKCδ wild-type mice. In PKCδ knockout mice no induction of Iba1 or iNOS was apparent suggesting that the inflammatory response was substantially reduced following LPS stimulation (Fig. 8 B,C) in these mice in concordance with the behavioral studies and the rapid recovery of these mice from LPS-induced endotoxemia (Fig. 7).

**PKCδ knockout mice have reduced cytokine responses, microglial activation and oxidative stress markers in the MPTP model of PD**

To validate our hypothesis that PKCδ can regulate microglial activation and the neuroinflammatory response, we utilized the well established MPTP mouse model in which

microglial activation and neuroinflammation is well documented (Wu et al., 2002; Wu et al., 2003; Benner et al., 2004; Przedborski et al., 2004; Hu et al., 2008; Brochard et al., 2009; Chung et al., 2010). Based on our results from the primary microglia studies in which PKCδ knockout microglia (PKCδ -/-) had reduced cytokine and chemokine levels, nitric oxide generation and lower levels of intracellular ROS, we reasoned that the microglial neuroinflammatory response would be reduced in the MPTP model of PD. The dramatic recovery of the PKCδ knockout mice from LPS treatment and the reduced neuroinflammatory responses in the substantia nigra also support this hypothesis. Both wild-type and PKCδ knockout mice were treated according to the acute MPTP model (4 doses of 18 mg/kg MPTP at 2 h intervals) and the neuroinflammatory responses were studied 24 h later the time point at which robust neuroinflammation was evident (Czlonkowska et al., 1996; Du et al., 2001; Wu et al., 2003; Przedborski et al., 2004). MPTP intoxication induced the mRNA levels of TNFα, IL-1β and iNOS as expected in the wild-type mice. In the PKCδ knockout mice however the MPTP-induced cytokine production was significantly reduced (Fig.10A) similar to the results obtained with the LPS-injected mice (Fig.9A). The microglial NADPH oxidase complex has been shown to be critical for microglial neurotoxicity and oxidative stress in the substantia nigra in the MPTP model. The gp91-phox subunit of the NADPH complex has been shown to be induced in microglia during the nigral neuroinflammatory reaction following MPTP intoxication in this model (Wu et al., 2003). We compared the levels of gp-91 phox between wild-type and PKCδ knockout mice injected with MPTP 24 h after treatment by Western blotting with nigral lysates. In wild-type mice injected with MPTP there was a strong induction of gp-91 phox as expected, however, in the

PKCδ knockout mice there was no detectable induction of gp91 phox (Fig. 10B). We also probed for microglial activation using the microglial marker Iba1. In MPTP-treated wild-type mice, we observed a typical robust microglial activation response in the substantia nigra as evidenced by the increased density and ramified morphology of the microglial cells (Fig. 10C, lower panel). In PKCδ knockout mice, however, reduced microglial activation morphology and microglial cell density was apparent suggesting that PKCδ could be crucial to microglial activation following MPTP-induced dopaminergic damage. Together, these results suggest that the microglial neuroinflammatory response is attenuated in PKCδ knockout mice. Sustained microglial ROS generation is believed to be a major mechanism contributing to the progressive degeneration of dopaminergic neurons in PD. Since the gp-91 phox and iNOS are the primary sources of microglial ROS in the MPTP model and were both reduced in PKCδ knockout mice, we reasoned that the oxidative stress markers in the substantia nigra of these mice would be attenuated following MPTP treatment and that PKCδ knockout mice could be protected against MPTP-induced dopaminergic degeneration. Together these results suggest that the microglial neuroinflammatory response and the resulting oxidative stress induced by MPTP treatment are attenuated in the PKCδ knockout mice, indicating that PKCδ could be a crucial upstream regulator of these responses in vivo.

**PKCδ knockout mice are protected against MPTP-induced nigrostriatal dopaminergic degeneration**

Previous studies from our laboratory have demonstrated that PKCδ is highly expressed in dopaminergic neurons and can be proteolytically activated by caspase-3 cleavage during oxidative stress-induced apoptosis (Anantharam et al., 2002; Kanthasamy et

al., 2003; Kaul et al., 2003; Zhang et al., 2007b). Importantly, the pharmacological inhibition of PKCδ using the chemical inhibitor rottlerin is neuroprotective in the MPTP model of PD. Our exciting new results in this study suggest that PKCδ can regulate the microglial neuroinflammatory response in vitro and in vivo. Since microglial neuroinflammation and oxidative stress have been shown to exacerbate dopaminergic degeneration in the nigrostriatal system, we determined if the PKCδ knockout mice are protected against nigrostriatal dopaminergic degeneration in the MPTP model. Age-matched wild-type and PKCδ knockout mice were treated with MPTP and nigrostriatal dopaminergic degeneration was assessed 7 days later. Striatal dopamine levels and its metabolites DOPAC and HVA were quantified by HPLC. As expected, MPTP intoxication caused a severe loss of dopamine and its metabolites in the wild-type C57 black mice at 7 days to about 25% of the saline-treated controls. In PKCδ knockout mice however, dopamine and its metabolites were reduced only by about 40-50% compared to saline-injected control mice (Fig 7 A, B, C). Statistically significant differences were observed between the PKCδ +/+ and PKCδ -/- mice injected with MPTP for dopamine ($p < 0.001$), DOPAC and HVA ($p < 0.05$). We also used TH-DAB immunostaining and stereological counting to accurately estimate the loss of dopaminergic neurons and striatal fiber density in the nigrostriatal system of in wild-type and PKCδ knockout mice. Both striatal TH fiber density (Fig. 11B) and the number of TH-positive immunostained dopaminergic neurons in the substantia nigra (Fig. 11C) were higher in PKCδ knockout mice treated with MPTP compared to wild-type mice demonstrating robust protection of the nigrostriatal system in PKCδ knockouts. Together, these results confirm that PKCδ signaling can participate in dopaminergic degeneration by acting as a

proapoptotic kinase in dopaminergic neurons as we have shown previously, and also by regulating microglial activation and proinflammatory responses as we have shown here for the first time.

**PKCδ knockout mice are protected against MPTP-induced motor deficits**

Motor impairments arising from nigrostriatal degeneration and dopamine depletion are a hallmark of PD in humans and can be recapitulated in the mouse MPTP model. We used the VersaMax Analyzer and rotarod performance tests to assess the motor deficits in the wild-type and PKCδ knockout mice 7 days after MPTP treatment, the time point at which the nigrostriatal lesion is apparent. We quantified several behavioral parameters including total rest time, horizontal activity, vertical activity, total distance traveled, total movement time and the number of stereotypy movements over a 10 min interval in saline- and MPTP-treated wild-type and PKCδ knockout mice. The total rest time (Fig. 12C) was significantly increased in MPTP-treated PKCδ wild-type mice but not in PKCδ knockout mice. Significant improvement in motor and behavioral function was obvious in the PKCδ knockout mice treated with MPTP compared to the wild-type as seen with the quantified horizontal activity, vertical activity, total distance traveled, total movement time and the number of stereotypy movements (Fig.12 C – G). The improved motor functions was also apparent with the rotarod performance test where MPTP-treated knockout mice were almost as competent on the rotarod as the saline controls with no statistical differences in the amount of time spent between the two groups. In contrast the MPTP-injected wild-type mice had the lowest amount of time recorded on the rotarod indicative of significant motor impairments arising from nigrostriatal degeneration. Together, these results confirm that the PKCδ

knockout mice are protected against MPTP-induced nigrostriatal dopaminergic degeneration and that the robust neuroprotection observed translates into improved motor and behavioral functions.

**Discussion**

Reactive microgliosis is well documented in human PD patients and all animal models of the disease. Although mechanistic studies over the last decade in animal models of the disease support a proinflammatory and neurotoxic role for chronically activated microglia in dopaminergic degeneration, the signaling pathways regulating microglial activation have not yet been established. Microglial NADPH oxidase has been shown to be a major source of ROS in the substantia nigra that can contribute to the loss of dopaminergic neurons (Wu et al., 2002; Wu et al., 2003; Qin et al., 2005). Similarly, the NFκB pathway has been shown to be an important regulator of the microglial proinflammatory response in the substantia nigra with the selective inhibition of NFκB being neuroprotective in the MPTP model (Ghosh et al., 2007a). The NFκB signaling pathway could be particularly relevant since it can regulate both the microglial pro-inflammatory response by modulating key cytokines such as TNFα and by regulating the levels of the oxidative stress generating components including subunits of the NADPH oxidase complex and iNOS. However, the intracellular signaling pathways that regulate the potentially neurotoxic proinflammatory responses of microglia in PD remain to be elucidated.

Our results in this study identify the PKCδ signaling pathway as a novel regulator of the microglial activation response both in vitro and in two animal models of PD that are

relevant to neuroinflammation. Importantly, our studies show that the PKCδ gene and protein levels are induced during microglial activation by distinct mechanisms and stimuli including TNF receptors (TNFα), TLR-4 (LPS) and phagocytosis (aggregated α synuclein). We also observed induction of PKCδ protein levels in BV-2 microglial cells by activated MMP-3, which could potentially activate microglia through protease activated receptors as suggested by previous reports (Kim et al., 2005; Kim et al., 2007). The significance of these findings is twofold. First, they suggest that PKCδ induction could be a common downstream signaling component involved in activation of microglial cells by distinct mechanisms and activation stimuli. The distinct subcellular localization pattern of PKCδ in primary microglia during activation by LPS, TNF and aggregated alpha synuclein supports this view. Secondly, our results suggest that the amount of PKCδ in the microglial cells could set the threshold and magnitude of the activation response. The increased mRNA and protein levels of PKCδ in activated microglia support this view, as do our results with shRNA knockdown of PKCδ expression levels in primary microglia and our results with PKCδ knockout mouse microglia. Further, our kinase assay results demonstrate that the increased PKCδ protein expression correlate with increased PKCδ kinase activity in microglial cells suggesting that the increased protein levels may be required to sustain the PKCδ signaling and maintain the activation state of the microglia. A recent study (Burguillos et al., 2011) reported proteolytic cleavage of PKCδ by caspase-3 in BV2 cells overexpressing PKCδ when activated with LPS. In our studies, we did not observe a proteolytically activated form of PKCδ in non-transfected BV2 cells or primary microglia stimulated with LPS, TNFα or aggregated α-synuclein but only an increase in the native protein which we found was expressed at low

levels in unstimulated microglia as reported by Burguillos et al. (2011). We posit that the proteolytic activation of PKCδ is likely an artifact of the overexpression system in the BV2 cells and does not occur under physiological conditions.

Our results with the multiplexed cytokine assays further support the involvement of PKCδ signaling in the microglial activation response by distinct mechanisms since significant changes were observed in the production of various cytokines and chemokines between microglia from PKCδ wild-type and knockout mice using three different activation stimuli (LPS, TNFα and aggregated alpha synuclein). More importantly, the levels of many cytokines and chemokines did not change between wild-type and PKCδ knockout mouse microglia indicating a selective and stimulus-specific regulation of the microglial cytokine response rather than an overall reduction in cytokine production or release. Significantly, our results showing reduced nitric oxide production and intracellular ROS generation in microglia isolated from PKCδ knockout mice demonstrate that PKCδ can regulate the microglial ROS generation machinery, which is particularly important in nigrostriatal dopaminergic degeneration and the progression of PD. Given that the p47 subunit of NADPH oxidase, the NFκB signaling pathway and iNOS are either direct substrates of PKCδ phosphorylation or are subject to regulation by PKCδ in other immune cells during proinflammatory events, it is likely that one or more these pathways is a downstream target of PKCδ signaling during microglial activation. Current studies in our laboratory are focused on identifying the downstream signaling pathways of PKCδ and its mechanism of activation in microglial cells.

The systemic LPS model was recently developed and has been used by several groups to investigate the mechanisms of chronic inflammation on dopaminergic degeneration (Qin et al., 2007; Lee et al., 2008). A hallmark of this model is the sustained elevation of TNFα levels in the brain that is evident even 10 months after a single systemic LPS injection (Qin et al., 2007). Our data demonstrates that in PKCδ knockout mice treated with LPS, the level of TNFα in the substantia nigra is not significantly higher than saline controls but is highly elevated (40- to 60-fold over saline controls) in wild-type mice similar to results obtained by shown by Qin et al. (2007). These findings are particularly relevant in the context of recent reports implicating a role for TNFα signaling in the progressive loss of dopaminergic neurons (Sriram et al., 2002; McCoy et al., 2006; De Lella Ezcurra et al., 2010), and suggest that PKCδ could be important in regulating both the systemic and CNS inflammatory responses. The reduced levels of Iba1 and iNOS in the substantia nigra of LPS-injected PKCδ knockouts compared to wild-type mice also support this view. Chronically activated microglia in the substantia nigra have been shown to produce proinflammatory mediators that can exacerbate dopaminergic degeneration by augmenting oxidative stress levels. Microglia NADPH oxidase is the primary source of free radicals in the nigrostriatal system and has been extensively investigated in animal models of PD. Further, therapeutic targeting the microglial response using anti-inflammatory strategies has shown efficacy in different animal models of the disease. Our results with the MPTP model demonstrate that the microglial inflammatory response and oxidative stress levels in the substantia nigra are reduced in PKCδ knockout mice. Significantly, both TNFα mRNA levels and the induction of the gp91-

phox subunit of the NADPH complex are attenuated in the knockout mice during the inflammatory response following MPTP treatment.

Our neuroprotection studies with the MPTP model demonstrate that the reduced microglial proinflammatory response in the PKCδ knockout can contribute to the protection of the nigrostriatal system from MPTP-induced damage. The preservation of the dopaminergic system in the PKCδ knockout mice also translates into improved behavioral and motor function. The robust neuroprotection seen in the PKCδ knockout mice is most likely a result of two independent functions of this kinase. One as a mediator of the microglial proinflammatory response by full length PKCδ proinflammatory signaling as we have shown here, and also by functioning as a proapoptotic kinase following proteolytic activation in dopaminergic neurons as shown previously (Anantharam et al., 2002; Kaul et al., 2003; Zhang et al., 2007a). Therefore the robust protection of the nigrostriatal system in the PKCδ knockout mice could be a combined effect resulting from ablation of PKCδ proinflammatory signaling in microglia and a resistance against oxidative stress-induced apoptosis in dopaminergic neurons. The reduced microglial inflammatory response in the PKCδ knockout mice results in lower levels of oxidative stress and proinflammatory mediators in the nigrostriatal system, and is likely to be a major factor contributing to the neuroprotection of the dopaminergic neurons in these mice. Conversely, the resistance to oxidative stress-mediated apoptosis in dopaminergic neurons is likely to result in less activation of the microglia thus mitigating the self-propelled cycle of dopaminergic degeneration and microglial activation, which is thought to drive sustained reactive microgliosis and neurotoxicity in the substantia nigra (Block et al., 2007).

A therapeutic rationale for targeting PKCδ is immediately apparent from these studies. Small molecule kinase inhibitors could potentially mitigate both the microglial proinflammatory responses and also directly protect against oxidative stress-mediated degeneration in dopaminergic neurons (Zhang et al., 2007a). The multifactorial etiology of disease pathogenesis and the complex genetic, environmental and cellular interactions which contribute to dopaminergic degeneration in PD make therapeutic intervention an enormous challenge (Sulzer, 2007; Obeso et al., 2010). Therefore, identifying therapeutic targets that are relevant across multiple modalities of disease pathogenesis will be necessary to effectively block disease progression. While oxidative stress, mitochondrial dysfunction and proteasomal impairment are causative factors that can trigger dopaminergic degeneration, reactive microgliosis and deregulated neuroinflammation can sustain this process and result in progressive neurotoxicity (Block et al., 2007; Whitton, 2007; Glass et al., 2010). Our novel findings presented here provide a rational for targeting PKCδ signaling to attenuate the microglial neuroinflammatory response and mitigate the progressive degeneration of dopaminergic neurons. In addition to the known pro-apoptotic functions of PKCδ during dopaminergic degeneration by oxidative stress, mitochondrial dysfunction and proteasomal impairment, our results here identify PKCδ signaling in microglia as a potential therapeutic target to block microglial neurotoxicity and progressive dopaminergic neuron loss.

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**Figure 1.**

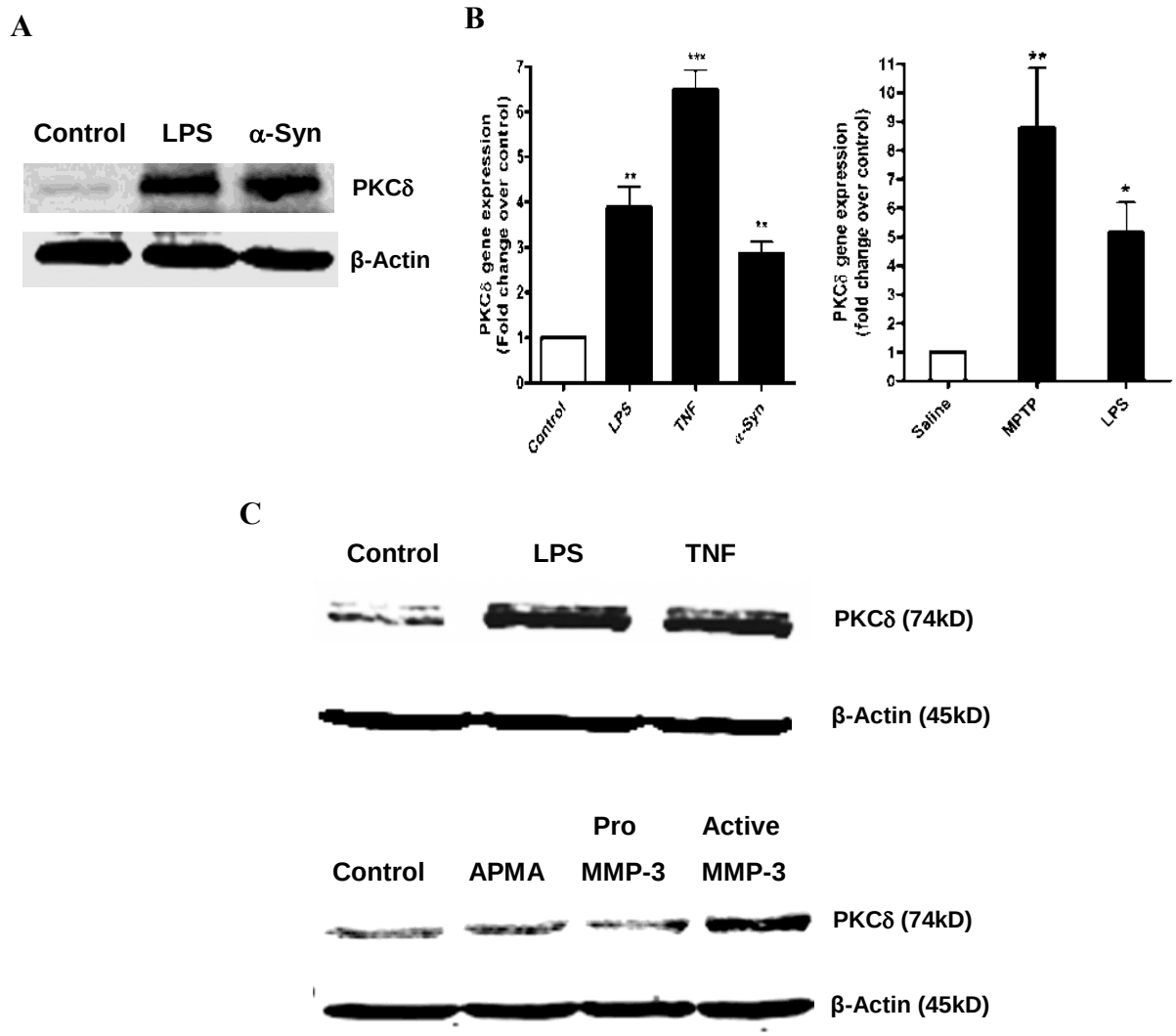



**Figure 1: Induction of PKCδ gene and protein expression during microglial activation.** **A,** Increased PKCδ protein expression in primary microglia. Purified primary microglia were treated with LPS (100 ng/ml) and 300 nM aggregated alpha synuclein (α Syn) for 24 h and PKCδ protein levels were probed by Western blotting using an antibody against the C-

terminus of the protein that detects both the native and cleaved fragments. **B,** Increased PKCδ gene expression in primary microglia and in the mouse substantia nigra. SYBR-green real time quantitative PCR analysis for PKCδ gene expression in isolated primary microglial cells treated with LPS (100 ng/ml), TNFα (30 ng/ml) and aggregated alpha-synuclein (300 nM) for 24 h (left panel). The normalized fold increase in PKCδ gene expression over the control group was calculated by the $\Delta\Delta C_t$ method. Increased PKCδ gene expression in the substantia nigra of mice treated with MPTP (4 x 18 mg/kg ip) and LPS (5 mg/kg, ip) for 24 h (right panel), the time point at which robust microglial activation and neuroinflammation is evident. Data represent the mean ± SEM from three experiments, (*** $p$ <0.001, ** $p$ <0.01 and * $p$ <0.05). **C,** Increased PKCδ protein expression in activated BV-2 microglial cells**.** Immortalized BV-2 microglial cells were activated with LPS (1 μg/ml) and TNFα (30 ng/ml) for 24 h and the PKCδ protein levels were determined by Western blotting. β-actin was used as the loading control (left panel).BV-2 cells were also treated with 250 ng/ml of pro and active MMP-3 enzyme for 24 h. The inactive proenzyme was activated using the organomercurial activator aminophenylmercuric acetate (APMA) in vitro. Increased PKCδ expression was observed with LPS, TNFα and active MMP-3 treatment in BV-2 cells. APMA alone did not increase PKCδ expression levels compared to untreated controls. Representative blots are shown and experiments were repeated at least three times.

**Figure 2.**

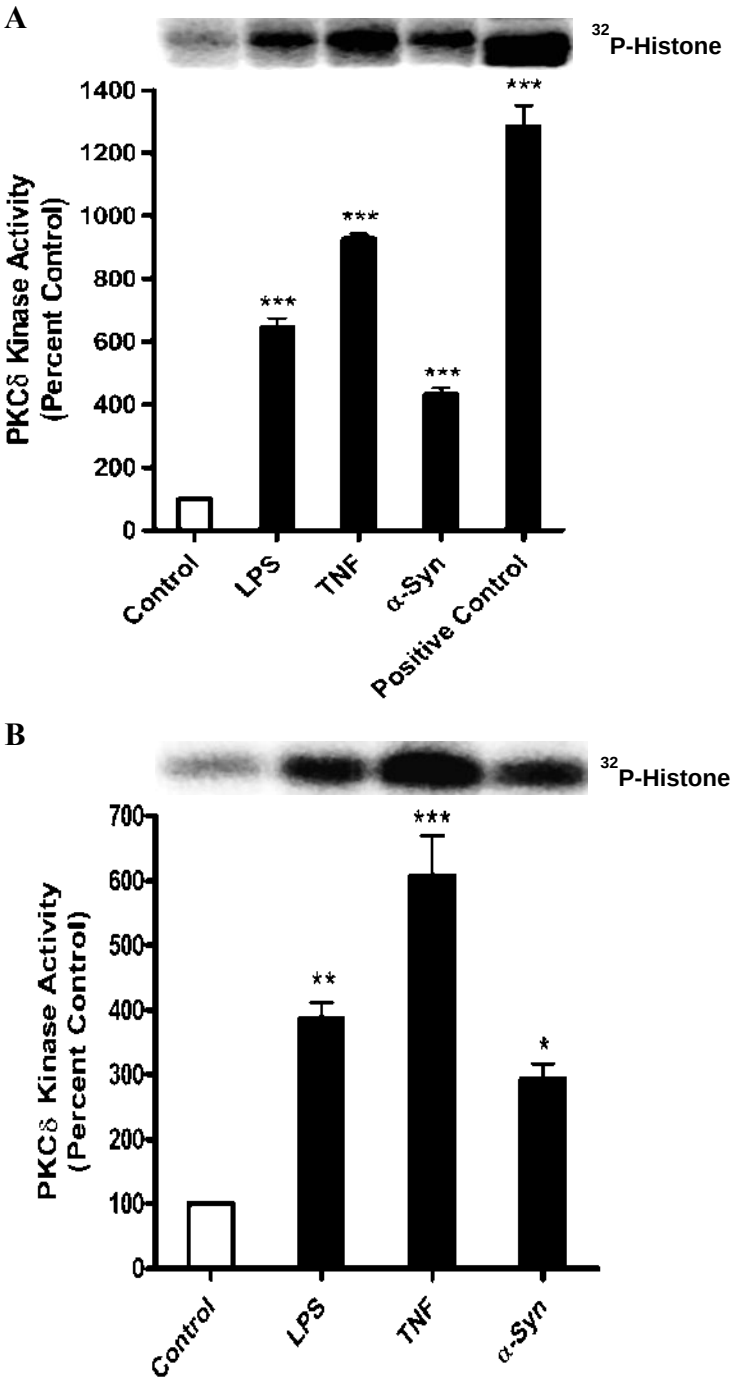



**Figure 2. PKCδ kinase activity is increased during microglial activation. A,** PKCδ kinase activity assay in primary microglia. The kinase activity of PKCδ was determined using a

standard immunoprecipitation-kinase assay. Primary microglial cells were treated with LPS (100 ng/ml), TNFα (30 ng/ml) and aggregated alpha synuclein (300 nM) for 24 h. The PKCδ protein was immunoprecipitated from 500 µg of total protein from each sample and processed for the in vitro kinase activity assay using a histone substrate and radiolabeled [γ-$^{32}$P] ATP. The kinase activity was quantified by densitometric analysis of the $^{32}$P-histone band intensity and expressed as percent control. The pure recombinant PKCδ protein was used as a positive control for the kinase assay. **B**, PKCδ kinase activity assay in BV-2 microglial cells. BV-2 cells were treated with LPS (1 µg/ml), TNFα (30 ng/ml) and aggregated alpha synuclein (300 nM) for 24 h and samples were processed for the PKCδ IP-kinase assay. In both BV-2 cells and primary microglia, the kinase activity of PKCδ is highly increased following microglial activation. Representative kinase assay gels are shown. Data represent the group mean ± SEM of densitometric values obtained from three independent experiments. Asterisks (***$p$<0.001, ** $p$<0.01 and * $p$<0.05) indicate significant differences between control and treatment groups

**Figure 3.**

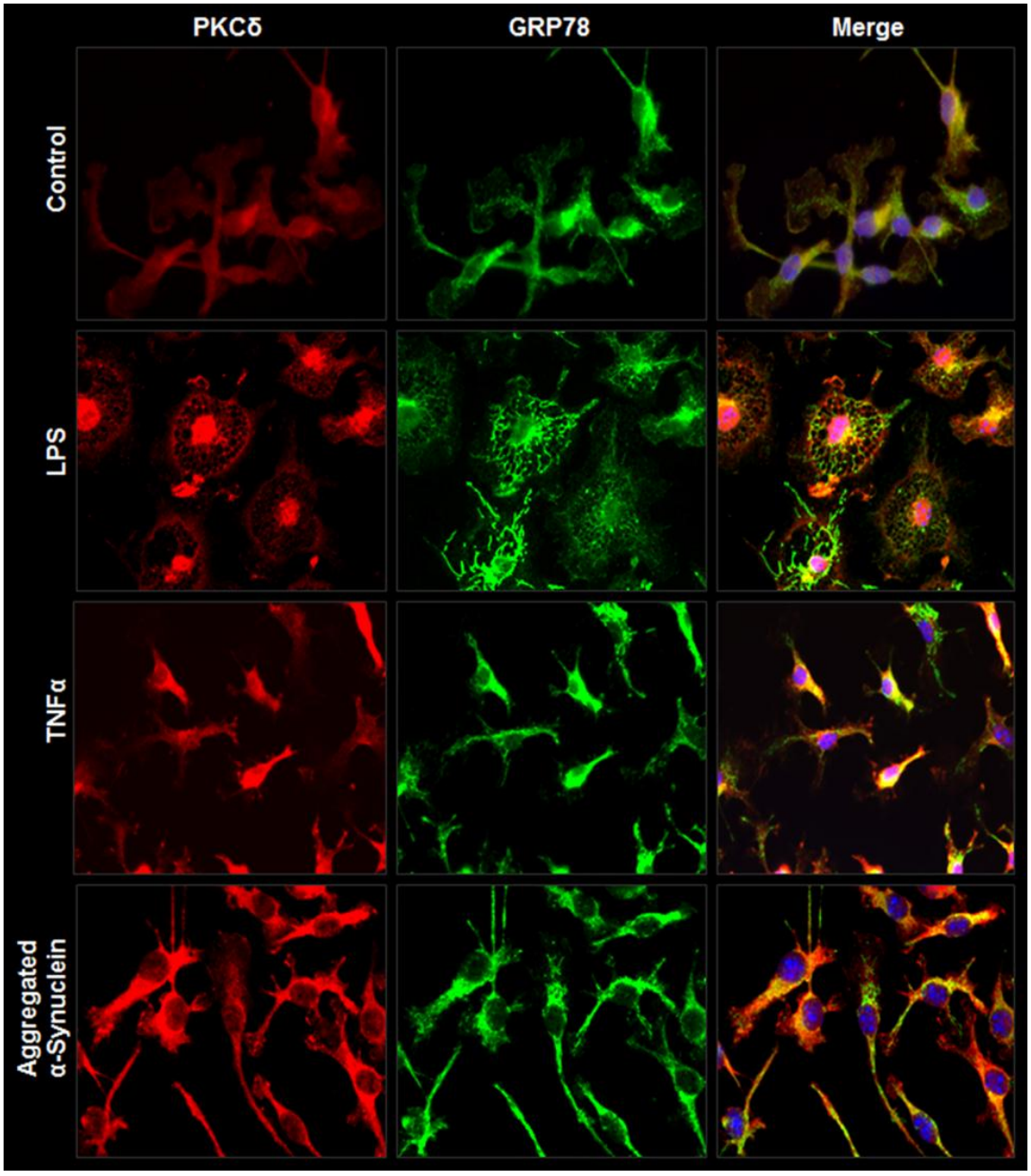



**Figure 3. Dynamics of PKCδ localization during microglial activation.** Isolated primary microglia were treated with LPS (100 ng/ml), TNFα (30 ng/ml) and aggregated alpha

synuclein (300 nM) for 24 h. Cells were fixed in 4% PFA and processed for immunofluorescence staining using a rabbit polyclonal antibody to PKCδ (Red, Alexa-555). The endoplasmic reticulum was labeled using the ER marker Grp-78 (Green, Alexa 488). The nucleus was labeled with Hoechst stain (blue). In control cells, diffused cytosolic staining of PKCδ is evident. LPS activated microglia showed the most dramatic changes in morphology and PKCδ localization with distinctive staining and accumulation in the nucleus and the ER network as seen in the merged images. In microglia activated with TNFα and aggregated alpha synuclein PKCδ staining was more distinct compared to controls but was largely excluded from the nucleus and showed more cytosolic accumulation. Experiments were repeated more than three times and representative images are shown.

**Figure 4.**

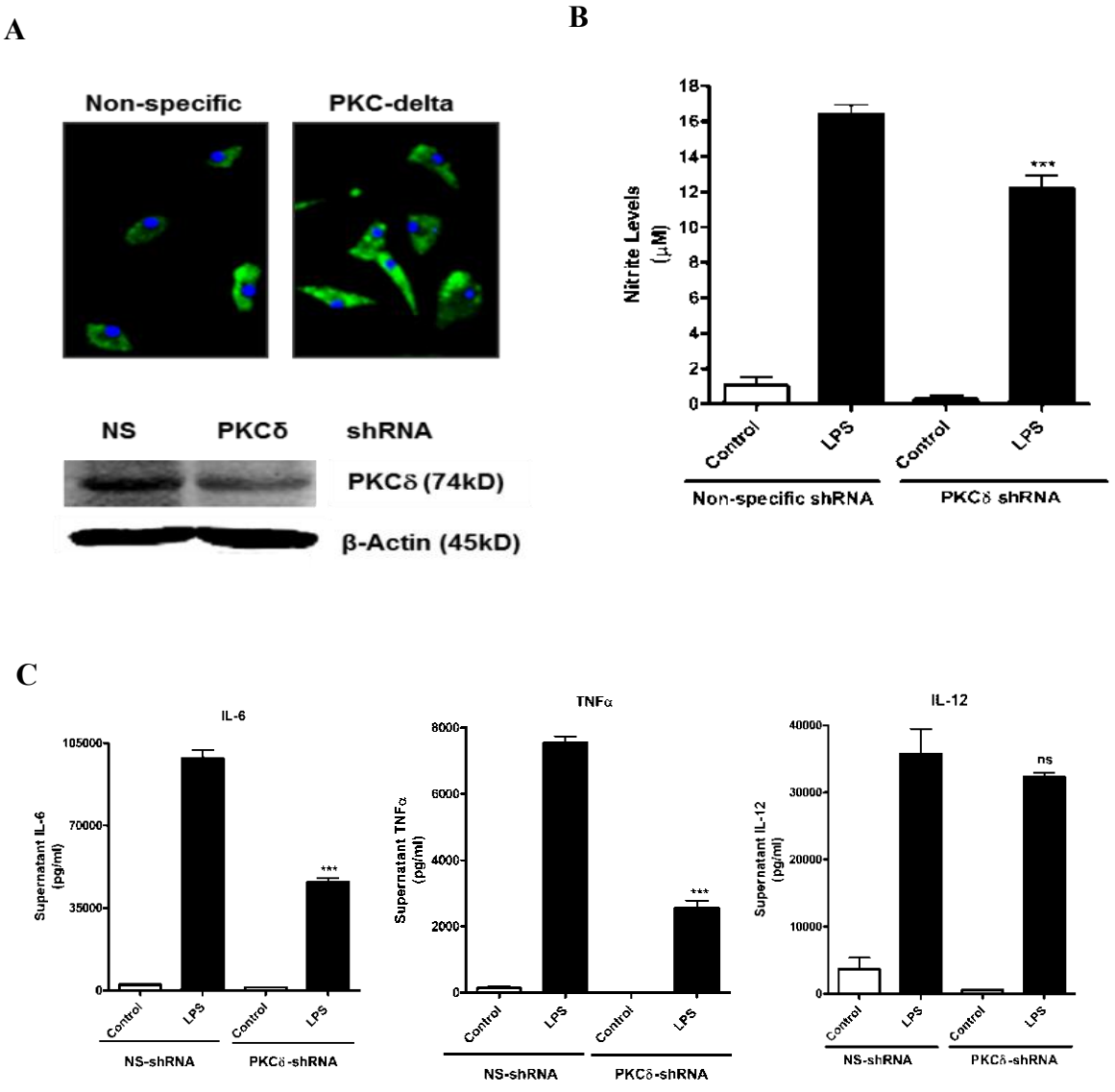



**Figure 4: Lentiviral sh-RNA knockdown of PKCδ attenuates LPS-induced microglial activation. A,** Isolated primary microglia were transfected with lentiviral vectors expressing either PKCδ sh-RNA or non-specific (NS) sh-RNA as controls. The transfections efficiency was determined by visualizing the GFP-tag co-expressed by the viral vectors and knockdown

of the PKCδ native protein level was confirmed by Western blotting. **B,** Knockdown of PKCδ attenuates LPS-induced microglial nitric oxide production. Primary microglia transfected with either non-specific or PKC-delta sh-RNA and were treated with LPS (100 ng/ml) for 24 h. Nitric oxide levels in the supernatant were determined using the greiss reagent. Primary microglial transfected with PKCδ sh-RNA showed reduced nitrite levels in the supernatant compared to microglia transfected with non-specific sh-RNA. **C,** Effect of PKCδ knockdown on LPS-induced pro-inflammatory cytokine production in primary microglia. Primary microglia transfected with either non-specific or PKCδ sh-RNA and were treated with LPS (100 ng/ml) for 24 h and pro-inflammatory cytokines were quantified using the luminex immunoassay system. Knockdown of PKCδ attenuated LPS-induced IL-6 and TNFα production but not IL-12 levels in the same supernatants. Experiments were repeated three times. Data represent the group mean ± SEM from one representative experiment. Asterisks (***$p$<0.001) indicate significant differences between non-specific sh-RNA and PKCδ sh-RNA transfected groups stimulated with LPS; "ns" denotes no significant difference.

**Figure 5.**

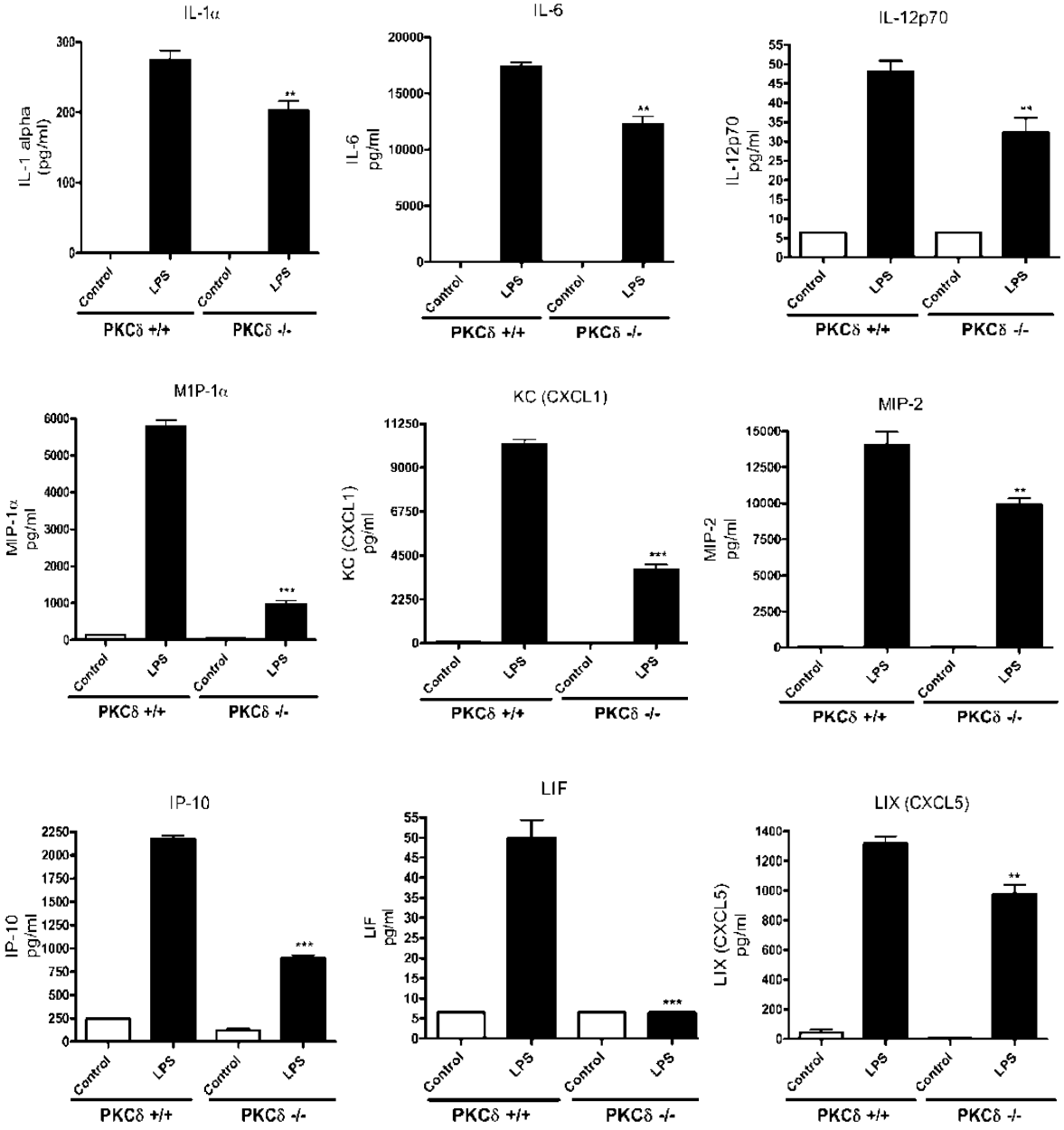



**Figure 5: Primary microglia from PKCδ knockout mice have attenuated cytokine and chemokine production in response to LPS stimulation.** Primary microglia were isolated from PKCδ wild-type (+/+) and knockout (-/-) mice and seeded in equal numbers on poly-D-

lysine coated plates. The cells were treated with LPS (100 ng/ml) for 24 h and cytokine levels in the supernatant were quantified using the Luminex immunoassay system. Primary microglia isolated from PKCδ knockout (-/-) mice showed significantly reduced levels of IL-1α, IL-6, IL-12p70, MIP-1α, KC (CXCL1), MIP-2, IP-10, LIF and LIX (CXCL5) upon LPS stimulation compared to microglia isolated from PKCδ wild-type (+/+) mice. Data represent the group mean ± SEM from three experiments. Asterisks (***$p$<0.001 and ** $p$<0.01) indicate significant differences between LPS-treated groups from PKC delta (+/+) and PKCδ (-/-) microglial cells (solid black bars).

**Figure 6.**

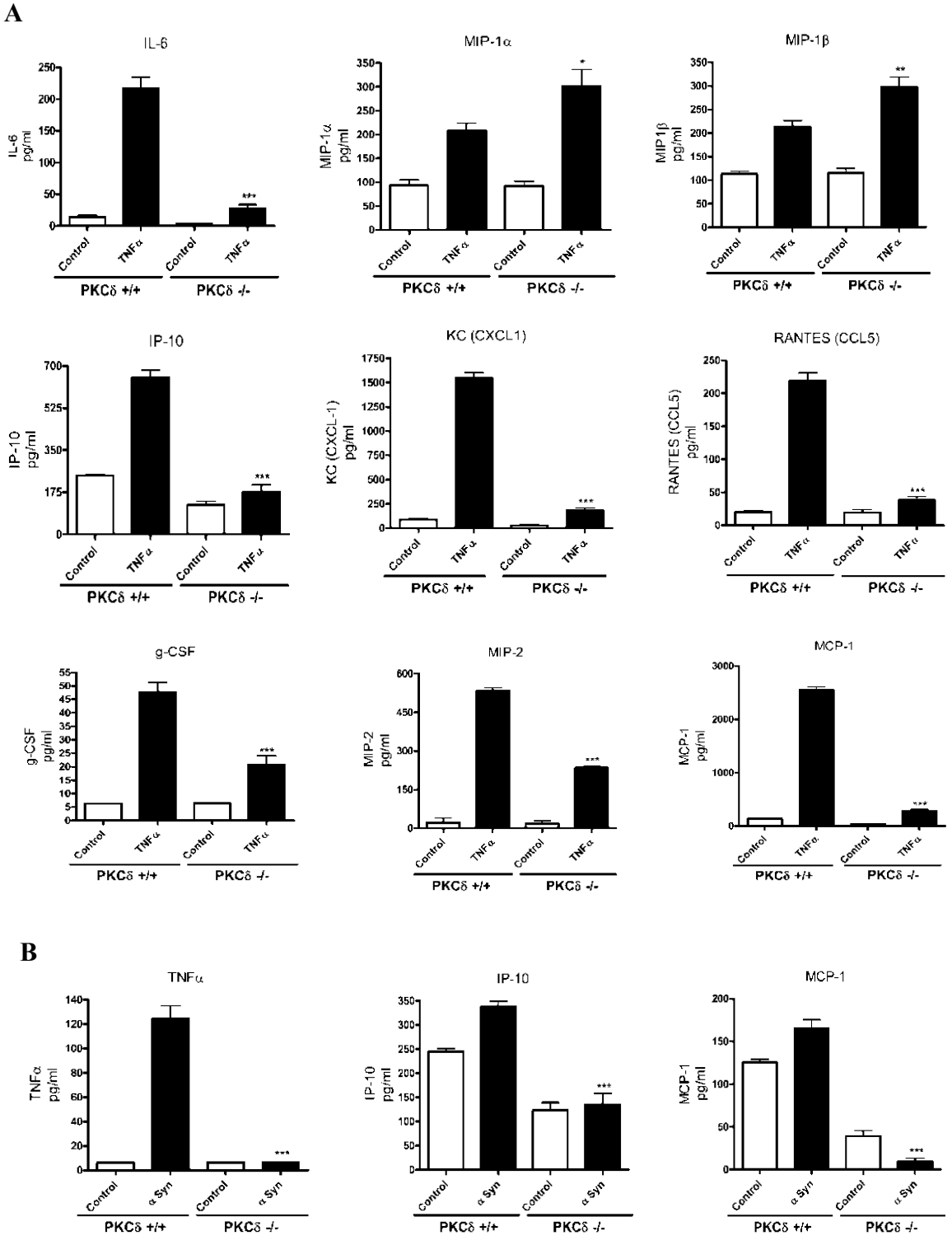


**Figure 6: Primary microglia from PKCδ knockout mice have attenuated cytokine and chemokine production in response to TNFα and aggregated alpha synuclein activation.** **A,** Primary microglia were isolated from PKCδ wild-type (+/+) and knockout (-/-) mice and seeded in equal numbers onto poly-D-lysine coated plates. The cells were treated with TNFα (30 ng/ml) for 24 h and cytokine levels in the supernatant were quantified using the Luminex immunoassay system. Primary microglia isolated from PKCδ knockout (-/-) mice showed significantly reduced levels of IL-6, IP-10, KC (CXCL1), RANTES (CCL5), g-CSF, MIP-2 and MCP-1 following TNFα stimulation compared to microglia isolated from PKCδ wild-type (+/+) mice. The levels of MIP-1α and MIP-1β however were significantly increased in the same supernatants. **B,** In primary microglia activated with aggregated alpha synuclein (300 nM for 24 h) only TNFα, IP-10 and MCP-1 were detected and the levels of all three were significantly reduced in PKCδ knockout mice. Data represent the group mean ± SEM from three to four experiments. Asterisks (***$p$<0.001, ** $p$<0.01 and $p$<0.05) indicate significant differences between LPS-treated groups from PKC delta (+/+) and PKCδ (-/-) microglial cells.

**Figure 7**

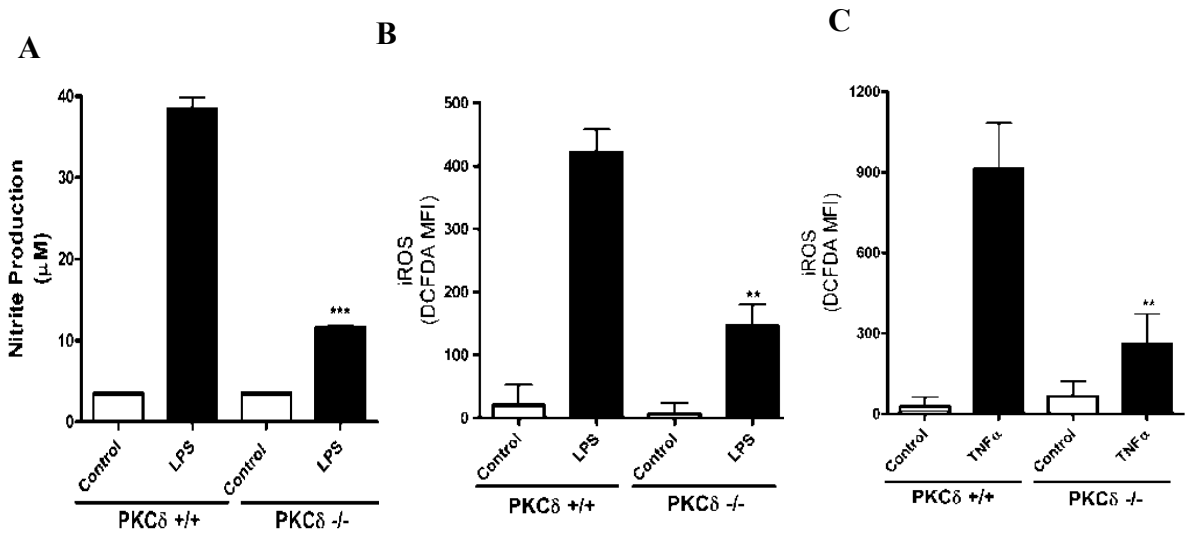



**Figure 7. Primary microglia from PKCδ knockout mice have reduced nitric oxide and ROS generation responses. A**, Nitric oxide assay. Primary microglia were isolated from PKCδ wild-type (+/+) and knockout (-/-) mice and treated with LPS (100 ng/ml) for 24 h. Nitric oxide production was determined the Greiss reagent and a sodium nitrite standard curve. Microglia isolated from PKCδ knockout mice showed reduced nitric oxide production compared to wild-type microglial cells. **B,** Reduced intracellular ROS generation in microglia from PKC knockout (-/-) mice. Primary microglia from PKCδ wild-type (+/+) and knockout (-/-) mice were treated with LPS (100 ng/ml, left panel) and TNFα (30 ng/ml, right panel) for 6 h and intracellular ROS generation was quantified using the fluorescent DCFDA dye. Microglia isolated from PKCδ knockout mice had attenuated intracellular ROS levels compared to those isolated from PKCδ wildtype (+/+) mice. Data represent the group mean ± SEM.Asterisks (***$p$<0.001 and ** $p$<0.01) indicate significant differences between LPS-treated groups or TNFα-treated groups from PKCδ (+/+) and PKCδ (-/-) microglial cells.

**Figure 8**

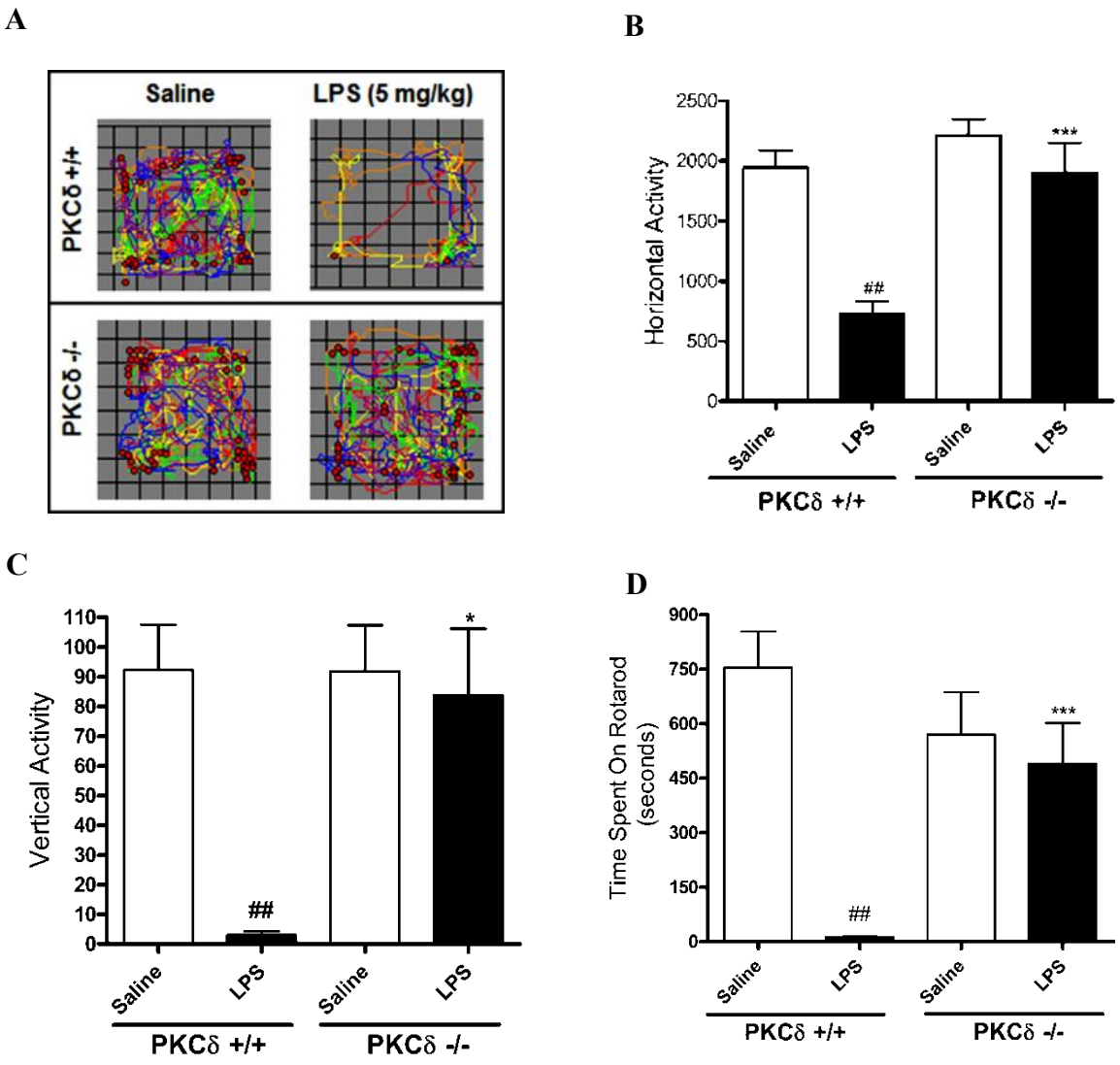



**Figure 8. Rapid recovery of PKCδ knockout mice (-/-) from LPS-induced endotoxemia and sickness behavior. A,** Locomotor activity and movement tracks (VersaPlot) of saline- and LPS-injected mice. Age-matched PKCδ wild-type (+/+) and knockout (-/-) mice were injected with a single dose of LPS (5 mg/kg, i.p.) or equivalent volumes of saline and the

behavioral activity was assessed 48 h later using the VersaMax Analyzer. The VersaPlot of the movement tracks demonstrates that LPS-injected PKCδ knockout (-/-) mice show almost normal locomotor activity similar to saline-injected controls (lower panel) as compared to PKCδ wild-type (+/+) mice (top panel). **B**, Total horizontal movements and **C,** Total vertical activity of saline and LPS-injected mice quantified using the VersaMax Analyzer 48 h after treatment showing significantly improved activity in PKCδ knockouts (-/-) compared to wild-type littermates injected with LPS. **D,** Rotarod performance analysis. The time spent on the rotarod was quantified 48 h after LPS (5 mg/kg) treatment. LPS-injected PKCδ knockout mice show improved performance on the rotarod compared to wild-type littermates. Data represent the group mean ± SEM from three to four experiments. Asterisks (***$p$<0.001 and ** $p$<0.01) indicate significant differences between LPS-treated groups from PKC delta wild-type (+/+) and PKCδ knockout (-/-) mice. ## denotes $p$<0.001 compared to saline injected mice.

**Figure 9.**

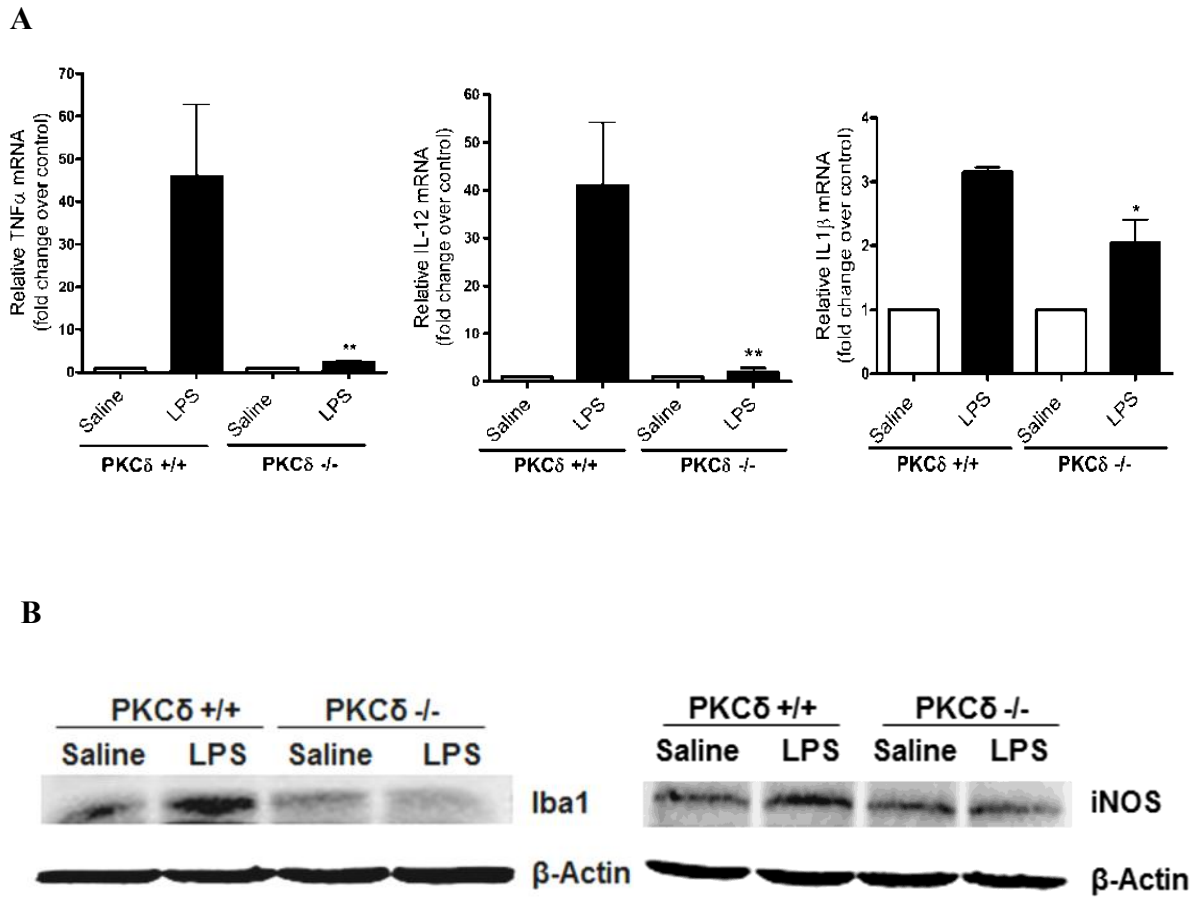



**Figure 9. PKCδ knockout mice have reduced nigral proinflammatory cytokine production and microglial inflammatory markers following systemic LPS treatment. A,** real-time SYBR green qRT-PCR for pro-inflammatory cytokines. Age-matched PKC delta wild-type (+/+) and knockout (-/-) mice were injected with 5 mg/kg LPS (ip) and proinflammatory cytokine gene expression was determined by real-time qRT-PCR 48 h later, the time point at which PKCδ knockout mice recovered form LPS toxicity based on behavioral studies. The levels of TNFα, IL-12 and IL-1β were highly expressed following

LPS stimulation in PKCδ wild-type mice but were significantly reduced in PKCδ knockout mice. Data represent the group mean ± SEM from three experiments. Asterisks (*$p$<0.05 and ** $p$<0.01) indicate significant differences between LPS-treated groups from PKC delta wild-type (+/+) and PKCδ knockout (-/-) mice. **B,** Western blot for Iba1 and iNOS. Nigral tissue was dissected from LPS-injected PKCδ wild-type (+/+) and knockout (-/-) mice 48 h after treatment and lysates were probed for the microglial activation marker Iba1 and for iNOS by Western blotting. PKCδ wild-type (-/-) mice had increased levels of Iba1 (left panel) and iNOS (right panel) following LPS treatment as expected and both markers were reduced in PKCδ knockout mice (-/-) suggesting that PKCδ has a critical role in regulating the microglial neuroinflammatory response in vivo following LPS stimulation. Representative blots are shown and experiments were repeated twice.

**Figure 10.**

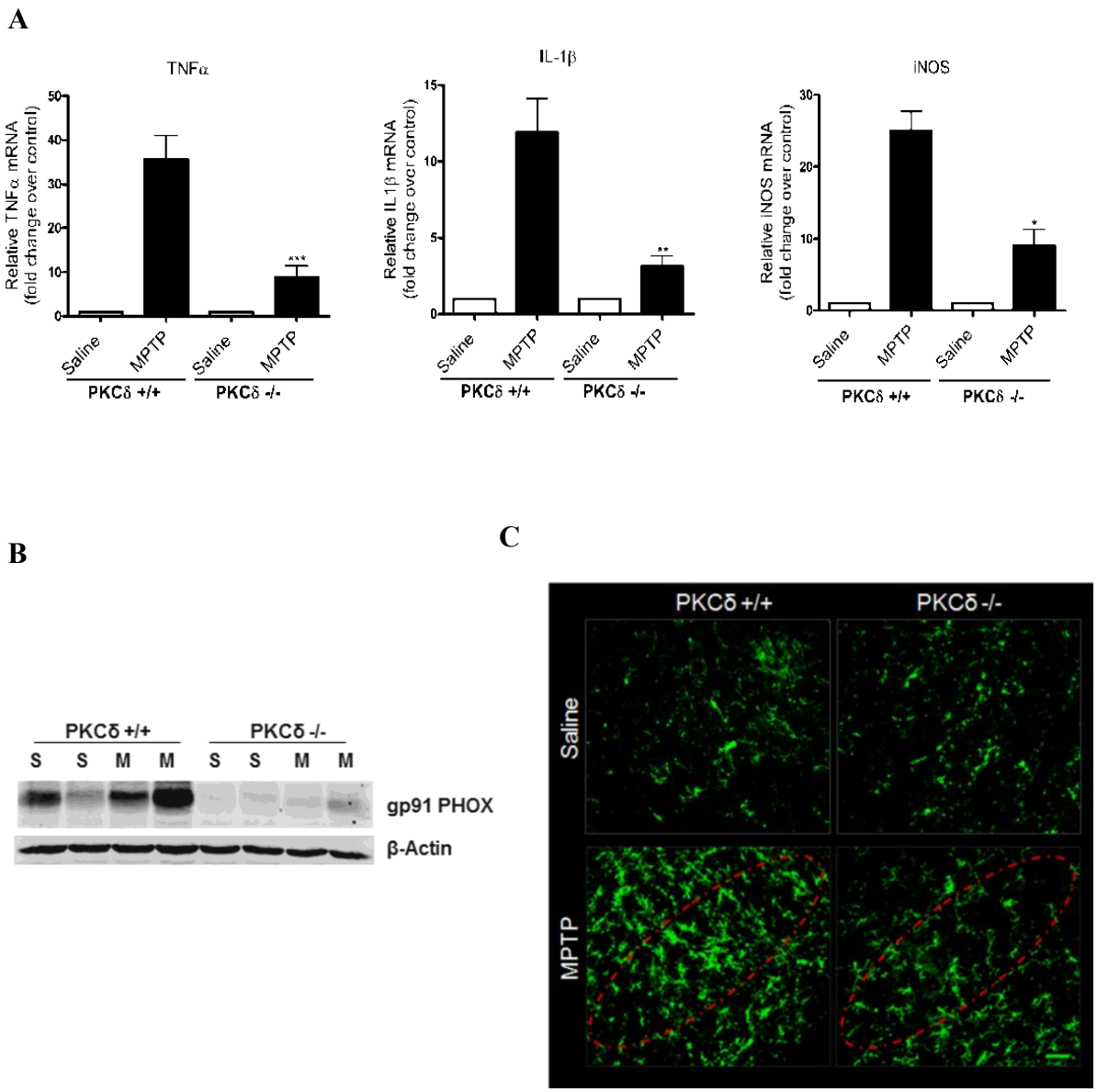



**Figure 10. PKCδ knockout mice have reduced nigral proinflammatory cytokine production, microglial activation and gp91PHOX levels following MPTP-induced neuroinflammation**. **A,** Real-time SYBR green qRT-PCR for pro-inflammatory cytokines in the mouse substantia nigra. Age-matched PKCδ wild-type (+/+) and knockout (-/-) mice

were injected with 4 doses of MPTP (18 mg/kg) or saline at 2 h intervals. Nigral tissues were dissected 24 h after the last injection and mRNA levels for TNFα, IL1β and iNOS were determined by real-time SYBR green qRT-PCR. The 18S rRNA gene was used as the internal normalizing control and the data expressed as fold change over saline-treated controls. Asterisks (*$p$<0.05, ** $p$<0.01 and ***$p$< 0.001) indicate significant differences between LPS-treated groups from PKCδ wild-type (+/+) and PKCδ knockout (-/-) mice injected with MPTP. **B,** Western blot for gp-91 PHOX. Nigral tissues from PKCδ wild-type (+/+) and knockout (-/-) mice were dissected 24 h after the last injection of either saline (S) or MPTP (M). Lysates were probed by Western blotting for gp91-PHOX, the membrane-bound subunit of the NADPH-oxidase complex, using a mouse monoclonal antibody. β-actin was used as a loading control. MPTP treatment caused a robust increase in gp91 PHOX protein levels in wild-type mice as expected but not i PKCδ knockout mice. Reduced basal levels of NADPH oxidase were also evident in PKCδ knockout (-/-) mice. Representative blots are shown and experiments were repeated three times. **C,** Iba1-immmunohistochemistry in nigral brain sections. Substantia nigra section from PKCδ wild-type (+/+) and knockout (-/-) mice were stained for the microglial activation marker Iba1 using a rabbit polyclonal antibody (1:500 dilution). Sections were then incubated with an Alexa-488 (green) conjugated secondary antibody. Images were captured using a Nikon TE-2000 fluorescence microscope at 20X magnification. The substantia nigra zone is outlined in red. Scale bar is 50 $\mu$m. Representative images are shown.

**Figure 11.**

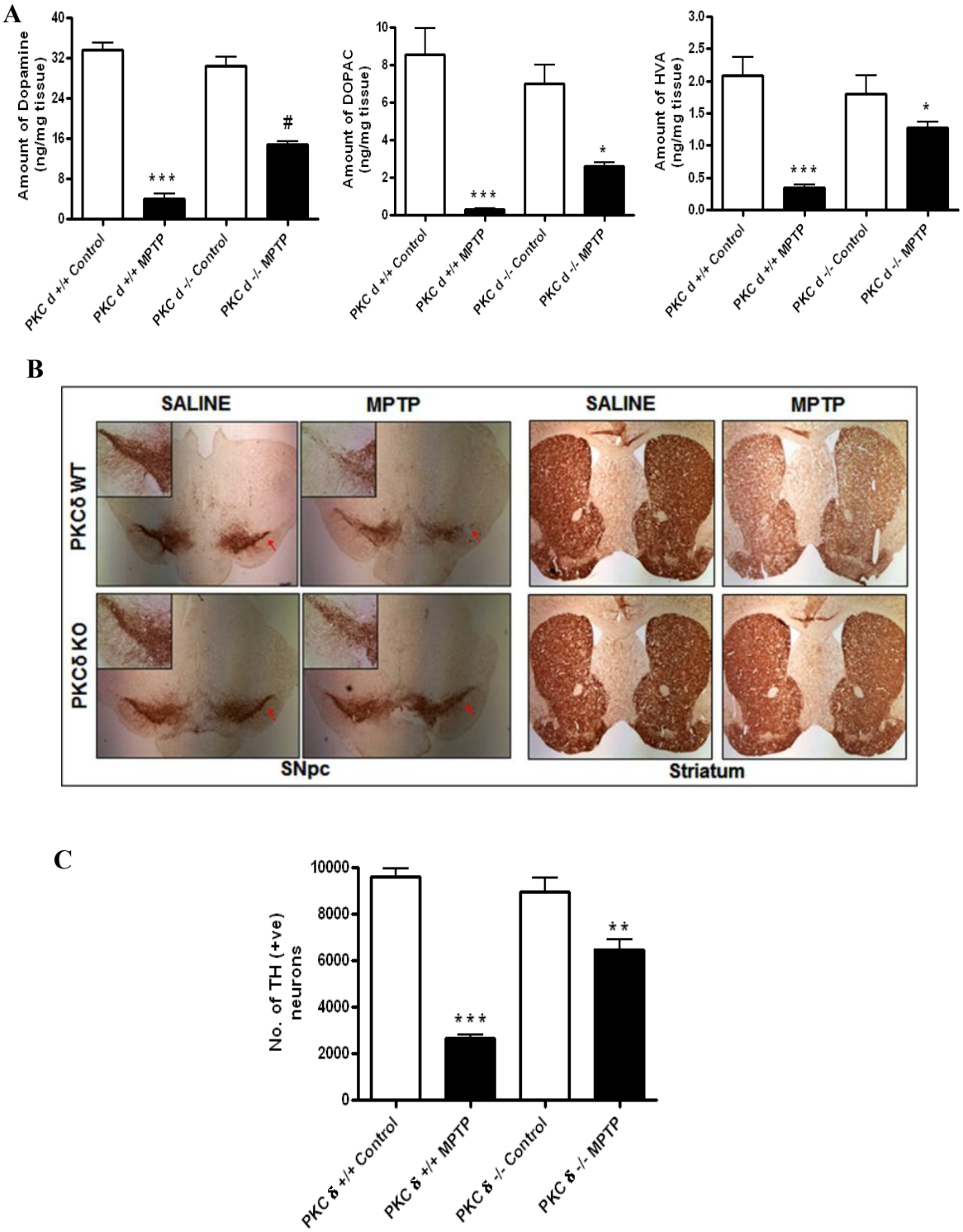


**Figure 11. PKCδ knockout mice are protected against MPTP-induced nigrostriatal dopaminergic degeneration.** **A,** HPLC analysis of striatal dopamine and its metabolites. PKC delta wild-type and knockout mice were treated with 4 doses of MPTP (18 mg/kg) or saline at 2 h intervals and were sacrificed 7 days after the last injection. The levels of striatal dopamine and its metabolites DOPAC and HVA were determined by HPLC. Data represent the mean ± SEM of six mice per group. Asterisks (***$p< 0.001$) indicate significant differences between saline and MPTP-treated wild-type mice; # $p<0.01$ and * $p<0.05$ denotes significant differences between PKCδ wild-type and knockout mice treated with MPTP. **B,** TH-DAB immunohistochemistry in substantia nigra and striatial sections. Mice were perfused 7 days after MPTP dosing and sections were processed for TH-DAB immunostaining. Images were captured at 2X and 20X (Inserts) magnifications for SNpc sections. Striatal sections were imaged at 2X magnification. PKCδ knockout mice were significantly protected against TH-neuron and terminal degeneration compared to wild-type littermates. Representative images are shown and experiments were repeated three times. **C,** Stereological analysis of TH-positive neurons in the substantia nigra of PKC wild-type and knockout mice following MPTP treatment.

**Figure 12**

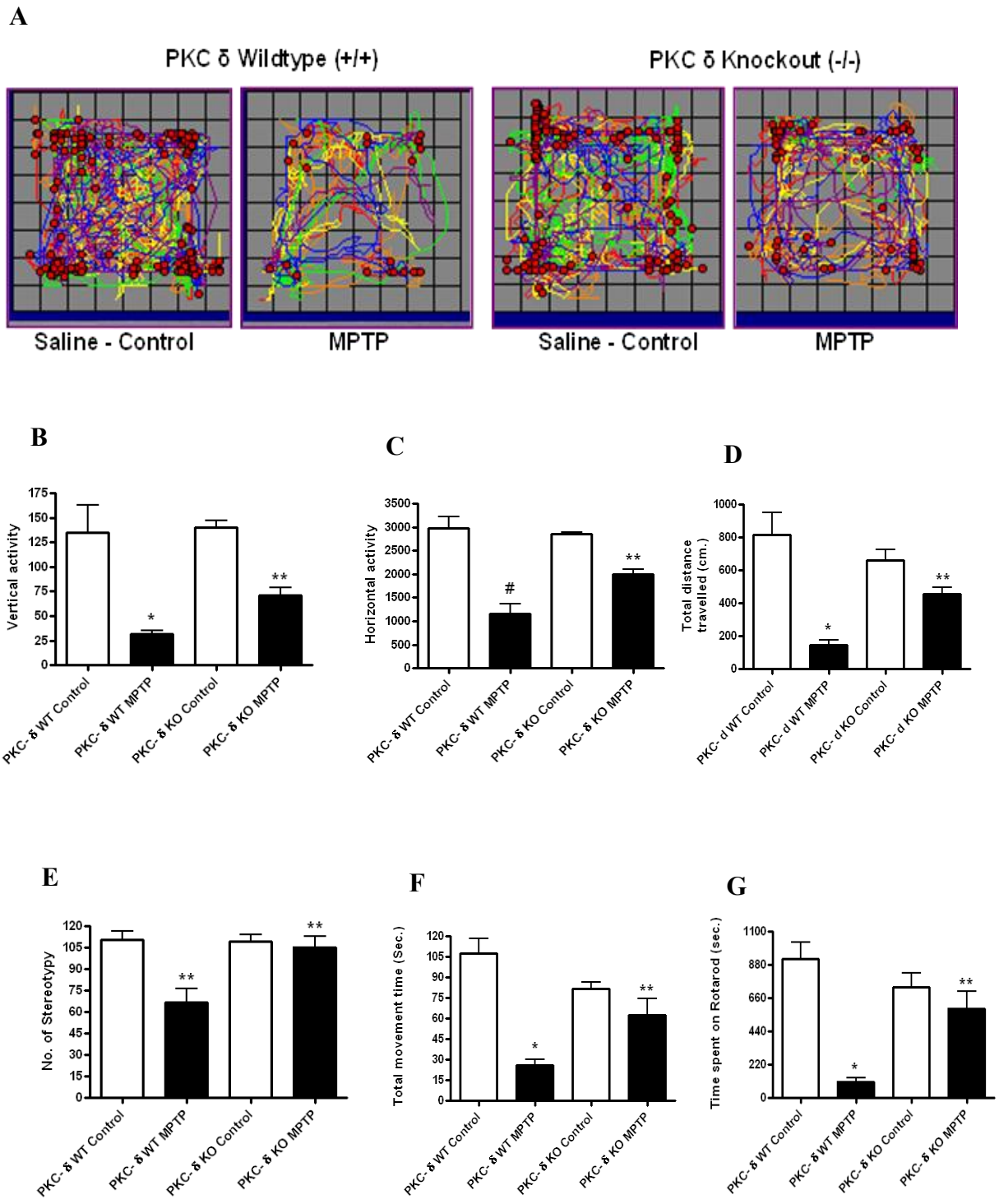


**Figure 12. PKCδ knockout mice are protected against MPTP-induced behavioral and locomotor deficits**. **A**, Versaplot movement track of mice. Locomotor and behavioral parameters were analyzed 5 days after MPTP treatment in PKCδ wild-type and knockout mice. Locomotor activity was measured using a VersaMax Analyzer. The movement track (Versaplot) of the mice collected over 10 minutes was plotted. Red dots denote vertical movements. B-F, quantification of locomotor and behavioral parameters using the VersaMax Analyzer. Saline or MPTP-treated mice were placed in the VersaMax Analyzer for 10 minutes and various locomotor activity parameters were measured simultaneously. PKCδ knockout mice showed significantly improved vertical activity **(B)**, horizontal activity **(C),** total distance travelled **(D),** number of stereotypy movements **(E)** and total movement time (F). G, Rotarod performance analysis. Rotarod analysis was performed 5 days after MPTP treatment and the amount of time spent on the rotarod was measured in PKCδ wild-type and knockout mice. Data represent the mean ± SEM of more than six mice per group. Asterisks (***$p < 0.001$ and ** $p<0.01$) denote significant differences between MPTP-treated wild-type (WT) and PKCδ knockout (KO) mice; # denotes a significant difference ($p<0.01$) between wild-type controls and MPTP-treated groups.

## Manuscript Supplementary Figures

**Figure 1. Electron microscopy imaging of alpha synuclein aggregates**

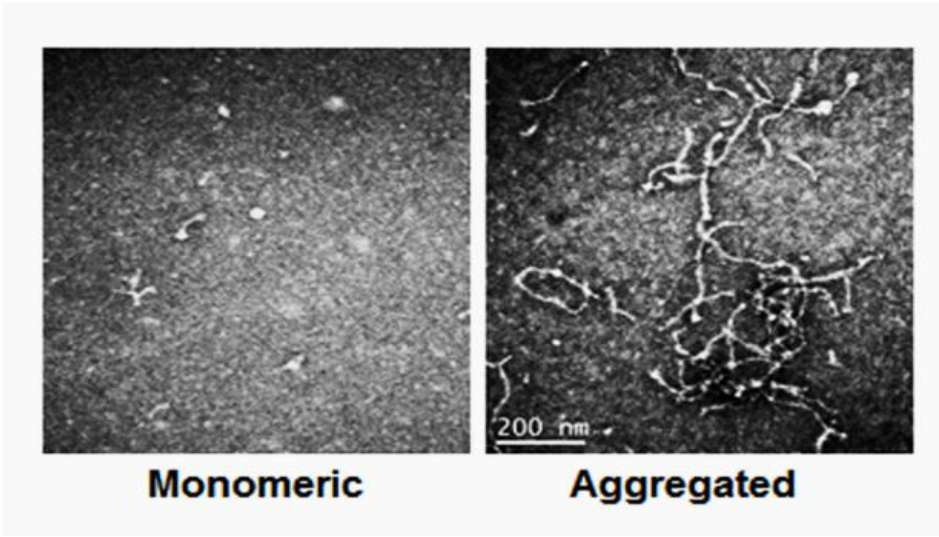
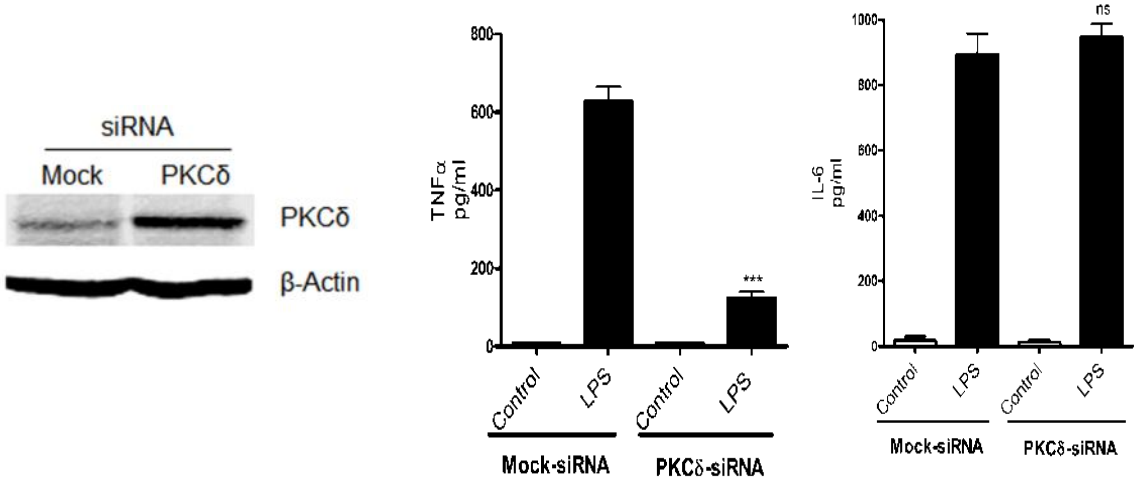



**Figure 2. Knockdown of PKCδ expression by siRNA attenuates LPS-induced TNFα levels**



# CHAPTER IV

## A SIMPLE MAGNETIC SEPARATION METHOD FOR HIGH-YIELD ISOLATION OF PURE PRIMARY MICROGLIA

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**Abstract**

Microglial cells play a dynamic role in the brain beyond their established function of immune surveillance. Activated microglia play a key role in neural development, neuroinflammation, neural repair and neurotoxicity. They are particularly important in several neurodegenerative diseases in which sustained microglial activation contributes to the progression of neurodegenerative processes. Consequently, understanding microglial function in CNS health and disease has become an area of active research in recent years. However, a significant obstacle to progress in this field has been the inherent difficulties in obtaining large amounts of primary microglial cells to routinely perform mechanistic studies and characterize the signaling pathways regulating the dynamics of microglial activation. Herein, we describe a novel column-free magnetic separation protocol for high-yield isolation of primary microglia from mouse postnatal mixed glial cultures. The procedure is based on optimized culture conditions that enable high microglial cell densities in the confluent mixed glial cultures followed by highly efficient recovery of pure microglia by

magnetic separation. The novel column-free magnetic separation system utilizes tetrameric antibody complexes (TAC) with dual specificity for the CD11b-PE labeled microglia and dextran magnetic nanoparticles. An FcR blocker (anti-CD16/32) is added to enhance the purity of the microglial separation by preventing non-specific labeling of other cell types. This procedure yields on average > 3 x $10^6$ microglial cells per mouse pup, with a remarkable purity of 97% and recovery of around 87% of microglia from the mixed glial population. Importantly, the microglia obtained by this method are fully functional and respond like cells obtained by conventional isolation techniques.

**Introduction**

The functional responses of microglia in the normal and diseased brain have become intensively investigated since their discovery as key players in the central nervous system (CNS). Besides their established functions of immune surveillance and phagocytic clearance in the brain, activated microglia have been shown to participate in neuronal survival by providing neurotrophic support and in maintaining neuron homeostasis during development (Bessis et al., 2007; Marin-Teva et al., 2004; Streit, 2002). They also have been implicated in facilitating neurogenesis and neural repair by directing the migration of neuronal progenitors to the sites of brain injury (Aarum et al., 2003; Walton et al., 2006). However, in chronic neurodegenerative diseases such as Alzheimer's or Parkinson's disease, there is compelling experimental evidence that microglial activation becomes deregulated and that the sustained reactive microgliosis that occurs can be neurotoxic and exacerbate neurodegeneration (Block et al., 2007). Under disease conditions, the persistently activated microglia can augment

neurodegenerative processes by promoting oxidative stress, as well as by producing proinflammatory mediators including cytokines and chemokines (Aschner et al., 1999; Glass et al., 2010)

Although much progress has been made at the cellular level in terms of defining the functional role of microglia as active players in CNS development, health and disease, far less is known about the subcellular processes, extracellular interactions and intracellular signaling pathways that regulate the dynamics of microglial activation. Particularly, the process by which microglia become chronically activated in neurodegenerative diseases and evade intrinsic mechanisms of regulatory control is largely unknown (Block et al., 2007; Glass et al., 2010). Understanding these phenomena would have immense therapeutic implications since reactive microgliosis is evident in most neurodegenerative diseases. The study of microglial cell biology, however, has been hindered due to inherent difficulties with obtaining large enough yields of primary microglial cells to routinely perform experimental techniques to elucidate signaling pathways, such as Western blotting, cell fractionation or proteomic studies. Unlike astrocytes, isolated primary microglia do not proliferate well unless specific growth factors are added. Immortalized cell lines, such as the BV-2 and N9 cells, have been widely used to study microglial responses. However, the highly dynamic and intricate process of microglial activation is poorly reproduced by such cell lines as they do not display the quintessential morphology changes seen with activation. Also, these cell lines have a far more limited cytokine and chemokine profile in comparison with primary microglia. *In vitro* experiments with isolated primary microglia are therefore of immense

utility in deciphering the signaling pathways and complex interactions regulating microglial activation and provide the most relevant model system for mechanistic studies.

Various protocols have been developed for microglial isolation. The differential adherence method is one of the most popular protocols. Mixed glial cultures are obtained from newborn rodent brains and allowed to grow to confluency. The microglial cells are then separated from the adherent astrocyte layer by agitation on a rotary shaker (Floden and Combs, 2007). The resulting microglial cell population, though highly pure, is low in number since most of the microglia that grow below the astrocyte layer are not recovered. Mild trypsinization has been reported as an alternative to the differential adherence method, and allows for a higher yield of pure microglial cells (Saura et al., 2003). Again, primary mixed glial cultures are obtained from newborn rodent brains and grown to confluency. An optimal concentration of trypsin, $Ca^{2+}$, and EDTA has been determined and used to selectively detach the astrocyte layer, leaving the microglial cells attached. Mild trypsinization allows for a higher yield of microglia with purities comparable to the differential adherence method, though the amount of microglial cells obtained is still insufficient when considering possible experimental designs. Column-based magnetic separation is one of the newer techniques for isolation of primary microglia (Marek et al., 2008). Confluent mixed cultures are trypsinized and incubated with CD11b magnetic microbeads that specifically label microglial cells. The labeled cells then are run through a magnetic column where the microglial cells are selectively retained, while unlabeled cells pass through and are removed. After several washes, a purified fraction of microglial cells can be eluted. The microglia yield from column-based magnetic separation, however, is no higher than that obtained with mild

trypsinization. With the advent of highly specific monoclonal antibodies that label unique cell populations, magnetic cell separation technology has progressed to become an indispensable tool for cell separation in the field of immunology, and has been widely used to separate and analyze almost every immune cell type (Grutzkau and Radbruch, 2010).

In this study, we report a new column-free magnetic separation method for high-yield isolation of primary microglia from postnatal mixed glial cultures, which is both simple and efficient. This procedure requires no special columns and can be carried out in routinely used round bottom polystyrene tubes. Unlike previously reported magnetic separation methods that utilize CD11b microbeads, our procedure utilizes bispecific tetrameric antibody complexes (TAC) and dextran-coated magnetic nanoparticles to label and purify microglial cells based on their selective expression of the CD11b epitope. Further, an anti-CD16/32 (Fcγ III/II receptor) antibody is added to the labeling reagent as an FcR blocker to enhance the purity of the microglial separation by preventing non-specific labeling of other cell types in the mixed glial population. This system provides a rapid and efficient separation of microglia with extremely high recoveries (87.98 ± 2.6%) and purity levels in excess of 97%, which is sufficient for most types of studies. The magnetically separated microglia show typical morphological and functional responses with various activation stimuli for all aspects of the microglial response that were tested, including nitric oxide production, cytokine release and intracellular ROS generation. Notably, this method affords a yield and recovery of primary microglial cells that is high enough to perform common biochemical experiments involving Western blotting, PCR, and proteomics without sacrificing unreasonable numbers of postnatal mouse pups.

## Materials and methods

### Preparation of mixed glia cultures

Primary cultures were prepared from C57bl6 postnatal day P0 to P1 mouse pups. Mouse brains were harvested, meninges removed and then placed in ice-cold Dulbecco's modified Eagle's medium/F-12 nutrient mixture (DMEM-F12,GIBCO Cat # 11320) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 U/mL penicillin, 50 μg/mL streptomycin, 2 mM L-glutamine, 100 μM non-essential amino acids, and 2 mM sodium pyruvate (Invitrogen). The tissue then was incubated in 0.25% trypsin (Sigma) in a 37°C water bath for 30 min with gentle agitation. Trypsinization was stopped by adding an equal volume of DMEM-F12 complete media, and the brain tissue was washed three times in the same medium. A single cell suspension of the brain tissue was prepared by gentle trituration and passed through a 70 μm nylon mesh cell strainer to remove tissue debris and aggregates. The cell suspension then was made up in DMEM-F12 complete medium and seeded such that one mouse brain was plated into 2 T-75 flasks, each containing a total volume of 12 mL. Cells were placed in a humidified $CO_2$ incubator at 37°C and allowed to attach. The medium was changed after 4 to 5 days and the mixed glial cells were allowed to grow to confluence. Microglial separations were performed on days 14 to 16, at which time the microglial cell density in the mixed glial cultures is high (Saura et al., 2003).

### Differential adherence isolation of microglia from mixed glial cultures

Confluent 14- to 16-day old mixed glial cultures were placed on an orbital shaker (Stovall Belly Dancer) at 120 rpm for 2.5 h to induce the detachment of the microglial cells. Immediately following agitation, the supernatant containing the microglia was collected and

centrifuged at 300 x g for 5 min and resuspended in 2 mL of fresh DMEM-F12 complete medium. The primary microglia were counted using a Beckman Coulter Vi-Cell XR automated cell counter and plated according to the cell number required for experiments.

**Column-free magnetic separation for isolating microglia from mixed glia**

Column-free magnetic separation was performed on 14- to 16-day old confluent mixed glial cultures using the EasySep mouse CD11b positive selection kit purchased from STEMCELL Technologies (Catalog # 18770). The principle of the column-free magnetic separation method is presented in Fig. 1. Cells were trypsinized with 3 mL of 0.25% trypsin per T-75 flask. The flasks were agitated on a rotary shaker for 20 min and the trypsin reaction was stopped by adding 6 mL of DMEM-F12 complete media. Any remaining adherent cells were scraped off using a cell scraper. The harvested cell suspension was then gently triturated to break down cell aggregates, and then passed through a 70 μm cell strainer to get a uniform single cell suspension. Obtaining a uniform single cell suspension, free from aggregates, is critical to achieve a high yield and purity with magnetic separation. The cells were centrifuged at 200 x g for 6 min and counted on a Beckman Coulter ViCell XR automated cell counter. The mixed glial cells were resuspended at a density of up to $1x10^8$ cells/mL in the recommended separation medium (PBS containing 2% FBS and 1 mM EDTA, with no calcium or magnesium) and transferred to a Falcon 5 mL polystyrene round-bottom tube (BD Biosciences, Catalog #352058). The labeling reagent containing the CD11b-Phycoerythrin (CD11b-PE) monoclonal antibody and the FcR blocker (anti-CD16/32) was added at 50 μL/mL. The cell suspension was mixed gently and incubated at room temperature for 15 min. The PE-Selection Cocktail (containing the bispecific tetrameric

antibody complexes directed against phycoerythrin and dextran) was then added at 70 µL/mL. The cell suspension was mixed well and allowed to incubate at room temperature for 15 min. The dextran-coated EasySep Magnetic Nanoparticles were gently mixed to obtain a uniform suspension and added at 50 µL/mL, mixed well, and allowed to incubate at room temperature for 10 min. The cell suspension was then made up to a total volume of 2.5 mL with separation medium. The cells were gently mixed to obtain uniform dispersion and the tube was placed into the EasySep Magnet obtained with the kit and set aside for 7 min. In one continuous motion, the EasySep Magnet was inverted to pour off the unlabeled cells. The magnetically labeled microglial cells remained inside the tube, held by the magnetic field of the magnet. The magnet and tube were left in the inverted position for 2-3 s, and then carefully returned to an upright position. The tube was then removed from the magnet and the magnetically retained cell suspension was made up to a volume of 2.5 mL with separation medium. The cell suspension was mixed by gentle pipetting up and down 2-3 times, and the tube was placed back into the magnet. The wash step was repeated 3 times to obtain purity levels of >97%. After the final wash, the purified microglial cells (positive fraction) were resuspended in DMEM/F12 complete media and counted using the Beckman Coulter Vi-Cell automated cell counter prior to seeding for experiments. The unlabeled cells obtained from the second and third pour-off steps were collected as the negative fraction.

**Flow cytometry**

The proportion of microglia in different cell populations was estimated by direct labeling with CD11b-PE followed by flow cytometric analysis. Cells were counted using a Vi-Cell XR automated cell counter and $1x10^6$ cells/mL were resuspended in 100 µL of flow

cytometry buffer (PBS with calcium and magnesium containing 0.01% sodium azide). Cells were labeled using the mouse CD11b-PE labeling reagent (STEMCELL Technologies) according to the manufacturer's instructions. After labeling, the cells were washed twice in flow cytometry buffer and resuspended at a final volume of 1 mL and stored on ice until analysis. Flow cytometry was performed on a BD FACSCanto flow cytometer at Iowa State University's centralized flow cytometry facility, and data analysis was performed using the instrument's software package.

**Immunofluorescence**

Immunofluorescence microscopy also was used to validate the purity levels of the microglial separations determined by flow cytometry. The positive and negative fractions obtained after separation, as well as the total mixed glial cell population obtained prior to magnetic separation, were seeded on poly-D-lysine- coated coverslips in a 24-well plate at a density of 200,000 cells per well. Cells were fixed with 4% paraformaldehyde for 30 min and washed five times with PBS. The cells were blocked and permeabilized for 1 h in blocking buffer (PBS containing 2% BSA, 0.5% TritonX and 0.05% Tween 20) and incubated with primary antibodies at 4°C overnight in PBS with 2% BSA. Primary antibodies used were goat polyclonal to Iba1 (Abcam) for microglia and rabbit polyclonal GFAP (Santa Cruz Biotechnology) for astrocytes, both at a concentration of 1:1000. After 3 washes in PBS, species-specific secondary antibodies (Alexa Fluor, Molecular Probes) were added at room temperature for 1 h. Images were captured using a Nikon Eclipse TE-2000 fluorescence microscope.

**Western blotting**

For Western blot analysis, magnetically separated microglia cells were plated at a density of 4 x $10^6$ cells per flask in poly-D-lysine coated T-25 flasks. Cells were stimulated with 100 ng/mL LPS or 200 nM aggregated alpha synuclein (rPeptide Inc.) for 24 h. After treatment, cells were collected by trypsinization and cell scraping. Cell pellets were lysed using RIPA buffer (Pierce Biotechnology) and 30 µg of protein was loaded per sample. Proteins were separated on 12% polyacrylamide gels and transferred to a nitrocellulose membrane (BioRad) overnight. Membranes were incubated in fluorescent Western blocking buffer (Rockland Immunochemicals) for 45 min and then probed with primary antibodies for gp91phox (BD Transduction) at a dilution of 1:1,000, iNOS/NOS2 (Santa Cruz Biotechnology) at a concentration of 1:200, and Iba1 (Abcam) at 1:1000. β-actin (Sigma-Aldrich) at a concentration of 1:10,000 was used as the loading control. Fluorescent infrared dye-tagged secondary antibodies (Molecular Probes) were used to visualize the bands, and the membranes were scanned on an Odyssey Infrared Imaging System (Li-Cor Biosciences).

**Microglial nitric oxide detection**

Microglial nitric oxide production was determined indirectly by quantification of nitrite in the supernatant using the Griess reagent (Sigma Aldrich). Magnetically separated microglia were plated in poly-D-lysine coated 96-well plates at 150,000 cells per well. After attachment, cells were treated with 100 ng/mL of LPS for 24 h or pretreated with 100 µM apocynin for 30 min. After treatment, 100 µL of supernatant was collected from each well and an equal volume of the Griess reagent was added. The samples were incubated on a plate shaker at room temperature for 15 min until a stable color was obtained. The absorbance at

540 nm was measured using a Synergy 2 multi-mode microplate reader (BioTek Instruments) and the nitrite concentration determined using a sodium nitrite standard curve.

**Cytokine assays**

Magnetically separated microglia cells were seeded in poly-D-lysine coated 96-well plates at 50,000 cells per well. Cells were treated for 24 h with 100 ng/mL of LPS or pretreated with 100µM apocynin for 30 min. After treatment, 50 µL of supernatant from each well was collected and frozen at -80°C. Cytokine levels were determined using the Luminex immunoassay system using recombinant cytokines as standards (Vignali, 2000).

**Intracellular reactive oxygen species (iROS) detection**

Intracellular reactive oxygen species (ROS) were determined using the fluorescent probe 2',7'-dichlorodi-hydrofluorescein diacetate (DCFH-DA) (EMD Chemicals), according to previously published protocols (Qian et al., 2007; Zhang et al., 2005). Purified microglia were plated on a 96-well plate at $10^5$ cells per well. Before treatment, cells were washed in HBSS and incubated with 40 µM DCFH-DA in HBSS containing 2% FBS for 30 min. Cells were then treated with 100 ng/mL LPS, 30 ng/mL of recombinant mouse TNF alpha (Sigma-Aldrich) or 200 nM aggregated alpha synuclein (rPeptide Inc.) for 6 h. For iROS inhibition studies with apocynin, cells were pretreated with 100 µM apocynin for 30 min.Shorter treatment times were chosen for iROS generation studies based on previously published reports (Zhang et al., 2005) and because maximal iROS generation occurs early during microglial activation. After treatment, the plate was incubated at 37°C for 1 h and the fluorescence intensity determined using a Synergy-2 multi-mode microplate reader at an excitation of 485/20 and an emission of 530/25. The fluorescence value from the control

cultures was subtracted as background and the increase in fluorescence with treatment was expressed as increased iROS (Qian et al., 2007; Zhang et al., 2005).

**Statistics**

The data are expressed as the mean ± SE. Statistical significance was determined by ANOVA, followed by Bonferroni t-test using the GraphPad Prism statistical analysis software package. Values of $p < 0.05$ were considered statistically significant.

## Results

### Yield and purity of primary microglia by magnetic separation

Flow cytometry and immunofluorescence studies were performed on parallel cultures to evaluate the purity of the microglia before and after magnetic separation. Confluent 14- to 16-day old mixed glial cultures grown in DMEM-F12 contained 25.55 ± 3.0% $CD11b^+$ microglia. After magnetic separation, the positive fraction contained 97.65 ± 0.83% pure $CD11b^+$ microglia and the negative fraction contained 1.650 ± 0.34% microglial cells (Table 1). Using flow cytometry and automated cell counting of the $CD11b^+$ population in the total mixed glial cells and magnetically enriched microglial fractions, we estimated the percent recovery. With differential adherence it was possible to recover only 25.94 ± 3.9% of the total microglial population from the mixed glial cultures. In comparison, our column-free magnetic separation procedure recovered about 87.98 ± 2.6% (Table 2). As depicted in Figure 2, the quantitative data obtained with flow cytometry were also confirmed by immunofluorescence using a different microglial marker, Iba1, which is commonly used to identify microglia. GFAP was used to label the astrocytes that were typically the most

abundant cells in the mixed glial cultures. Mixed cultures before magnetic separation showed distinctive populations of microglia and astrocytes (Fig.2A-B). Immunofluorescence labeling of positive fractions obtained after magnetic separation revealed a co-localization of almost all the Hoechst positive cells with Iba1 staining (Fig.2C-D), indicating that most of the cells are microglia. Conversely, the negative fractions contained mostly GFAP-positive astroglial cells (Fig.2E-F). In terms of purity, both magnetic separation and differential adherence had more than 97% microglial purity, as determined by immunofluorescence (Fig.3A to F) and quantification of the nuclear stain Hoechst co-localized Iba1-positive microglia and GFAP-positive astrocytes (Fig. 3G).

**Morphology, nitric oxide generation and cytokine release**

Since functional validation of the microglia is critical to establishing the utility of our method, we examined different aspects of the microglial response in the magnetically separated microglia following the activation with known stimuli. Phase contrast microscopy revealed the typical microglial morphology normally seen with activation, including hypertrophy and extensive branched processes, as compared to the unstimulated microglia (Fig. 4B-C). The morphology of the resting microglia showed slight variations depending on the cell density and the number of days *in vitro*. Next we studied the microglial response to the bacterial endotoxin LPS (Glezer et al., 2007; Lehnardt et al., 2003), and also used the well characterized NADPH oxidase inhibitor apocynin to attenuate LPS-induced activation (Bedard and Krause, 2007; Vejrazka et al., 2005). As shown in Fig. 5, nitrite levels were profoundly increased upon LPS treatment at 24 h and were attenuated with apocynin pretreatment. We also studied the cytokine responses from magnetically separated microglia

stimulated with LPS. The levels of two proinflammatory cytokines TNFα and IL-1β, as well as the prototypical anti-inflammatory cytokine IL-10, were measured in cell-free supernatants after 24 h of LPS treatment. As expected, LPS induced a dramatic increase in cytokine levels while apocynin treatment attenuated LPS-induced cytokine production to various extents depending on the specific cytokine (Fig. 6). The suppression of microglial nitric oxide production and cytokine release by apocynin is consistent with the established functions of apocynin as an inhibitor of peroxynitrite formation and cytokine production in microglia and other phagocytic cells (Stefanska and Pawliczak, 2008). These results demonstrate that the magnetically isolated microglia show expected activation profiles for both nitric oxide and cytokine production and have similar responses compared to primary microglia obtained by other methods.

**Intracellular ROS generation**

Rapid intracellular ROS generation by the NADPH oxidase complex is a critical regulator of microglial activation since it controls other aspects of the microglial response, including proinflammatory gene expression, rapid proliferation and the dynamic morphology changes seen in activated microglia. We used the fluorescent probe DCFH-DA to determine the production of intracellular ROS (iROS) in the magnetically separated microglia in response to alpha synuclein, LPS, and TNFα, which are known to induce iROS generation (Wang et al., 2004; Zhang et al., 2005). We observed significant increases in levels of iROS production with each of the stimuli at 6 h, with TNFα showing the highest levels of iROS (Fig. 7A). Again, apocynin pretreatment significantly attenuated LPS-induced intracellular ROS production (Fig. 7B), which would be expected based on its known function as a

NADPH oxidase inhibitor in immune cells including microglia (Peng et al., 2009). These results demonstrate that the magnetically separated microglia show the expected intracellular ROS responses upon activation by various relevant stimuli.

**Western blot analysis of magnetically separated microglia**

One of the current methodological challenges in microglial research is getting an adequate number of cells for biochemical investigations by Western blot. To demonstrate the versatility of the magnetic separation method for biochemical studies, we performed Western blot analysis in the magnetically purified microglia by examining typical signaling events associated with microglial activation. We probed the Western blot for the changes in expression of the gp91phox subunit of the NADPH oxidase complex as well as the inducible nitric oxide synthase (iNOS) and Iba1, which are known to be upregulated upon microglial activation. In concordance with our nitric oxide and iROS data, we observed increased levels of iNOS and gp91phox protein levels in activated microglia as well as a significant induction of Iba1 (Fig. 8A,B). It is important to note that the number of microglial cells required for such an experiment can be obtained from as few as 6 postnatal pups, based on the estimated yields obtained with our column-free magnetic separation protocol.

**Discussion**

We have developed a novel method to isolate primary microglia from postnatal mixed glial cultures by column-free magnetic separation technology based on tertrameric antibody complexes (TACS) and dextran-coated magnetic nanoparticles. The utility of our system lies in the high yield of microglia obtained (>3x$10^6$ cells/mouse pup), with purity levels (>97%) comparable to those obtained with current methods. This procedure is simple, quick and

reproducible, and requires no special columns or equipment other than the magnet obtained with the isolation kit. A significant constraint of currently available methods for microglial isolation from postnatal mixed glial cultures has been the low yield obtained, which limits the type and number of studies that can be conducted. Particularly, experiments that require large amounts of cells as starting material such as RNA isolation for quantitative PCR, Western blot analysis, cell fractionation or proteomic studies cannot be routinely performed with primary microglia using current methods because of the unreasonable number of postnatal mouse pups that would be required. Our method can overcome these limitations by providing more microglial cells for various biochemical experiments.

The most commonly used differential adherence or plate shaker method yields, at best, less than 1 x $10^6$ microglia cells per mouse pup (Floden and Combs, 2007; Marek et al., 2008; Saura et al., 2003). The trypsinization method developed by Saura et al. yields on average 1.75 x $10^6$ microglial cells per mouse pup, which is comparable to that obtained recently by Marek et al. (1.6 x$10^6$ cells per neonatal brain) using column-based magnetic separation with CD11b microbeads (Floden and Combs, 2007; Marek et al., 2008; Saura et al., 2003). Our protocol using column-free magnetic separation with tetrameric antibody complexes yielded on average >3 x $10^6$ cells per mouse pup, with more than 85% recovery of the primary microglia from the mixed glial cultures. We believe that the increased yield of microglia obtained with our procedure is the result of optimized conditions that facilitate a high microglial cell density in the mixed glial cultures, as well as the efficient recovery of the microglia by employing tetrameric antibody complexes in the magnetic separation steps. Our pilot studies to optimize our method demonstrated that age of the mouse pups, the medium

used for mixed glial cultures and the number of media changes were critical factors influencing the proportion of the microglial cells obtained with 14- to 16-day mixed glial cell cultures. We observed lower numbers of primary microglia and more astrocytes when DMEM was used instead of DMEM-F12, as suggested by other investigators (Saura, 2007). Consequently, lower yields of microglia were obtained with DMEM medium (supplementary figure 1). The precise reason for higher numbers of microglia in mixed glial cultures grown in DMEM-F12 is not known but is likely due to the additional supplements present in the F-12 fortified media being beneficial for microglial proliferation. We changed the mixed glial culture media only once at day 4 to 5 to allow for microglial growth factors produced by astrocytes such as colony stimulating factor-1 (CSF-1) to accumulate and promote microglial proliferation over the remaining culture period (Hao et al., 1990). As with all protocols for isolation of postnatal microglia, the age of the mouse pup is a crucial determinant of the final number of viable microglial cells obtained in mixed glial cultures (Giulian and Baker, 1986). The use of older mouse pups (P2 or later) usually results in significantly lower amounts of microglia in the mixed cultures and subsequently lower yields after magnetic separation. It is likely that the viability and proliferative capacity of microglia or their progenitor cells are considerably reduced in older mouse pups and could be the reason for the lower yields obtained. The yield and purity levels of primary microglia obtained by our method are high enough to provide sufficient numbers of cells for key experiments including qPCR analysis, Western blotting or proteomic studies which are otherwise problematic using conventional isolation protocols because of the inherently low yields obtained.

Functional validation of the microglial cells obtained by magnetic separation is crucial to demonstrate the feasibility of a new isolation technique. Therefore, we extensively tested the microglia obtained by magnetic separation using different functional assays, including cytokine production, nitric oxide release and intracellular ROS generation. The magnetically separated microglia show typical morphology changes associated with LPS stimulation and the expected increases in cytokine release, NO production and iROS generation. More importantly, the NADPH oxidase inhibitor apocynin effectively attenuated the LPS-induced cytokine release and ROS generation, demonstrating the utility of magnetically separated microglial cells for pharmacological studies. Aggregated alpha synuclein and TNFα treatments also increased intracellular ROS generation in these cells. The functional responses associated with microglial activation were also validated by Western blot analysis of the associated signaling proteins iNOS and gp91phox, which were induced during microglial activation along with Iba1. Collectively, these results demonstrate that the microglia obtained by our magnetic separation procedure retain their characteristics and function similarly to those isolated by conventional methods.

Isolation of microglia from adult mouse brain is a more representative model for understanding microglial function, particularly in neurodegenerative diseases where age is a critical factor. Indeed, some researchers have shown that adult brain microglia can react differently from those obtained from postnatal mice (Floden and Combs, 2006), while others have found only subtle differences (Moussaud and Draheim, 2010). However, because of the inherent difficulties in isolating microglia from the adult brain and the more complex protocols involved, adult brain microglia are still not as commonly used for *ex vivo* studies as

postnatal microglial cultures. Given that adult brain microglia express high levels of the CD11b epitope, the column-free magnetic separation procedure outlined here should be adaptable for separating microglia from adult mouse brain cell suspensions, similar to protocols used with CD11b microbeads (de Haas et al., 2007). We are currently optimizing the tissue processing steps in an attempt to develop a method to isolate adult brain microglia with this system.

In summary, we have developed and optimized a simple column-free magnetic cell separation technique for high-yield isolation of primary microglia from postnatal mixed glial cultures. The higher number of microglial cells obtained by this method allows for studies requiring large amounts of microglia to be performed more frequently. It should also reduce the number of animals needed, since more experiments can be done with fewer mouse pups as starting material. Given the increased interest in understanding microglial function in CNS health and disease, this method could be of immense utility for researchers studying microglial cell biology.

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**Figure 1.**

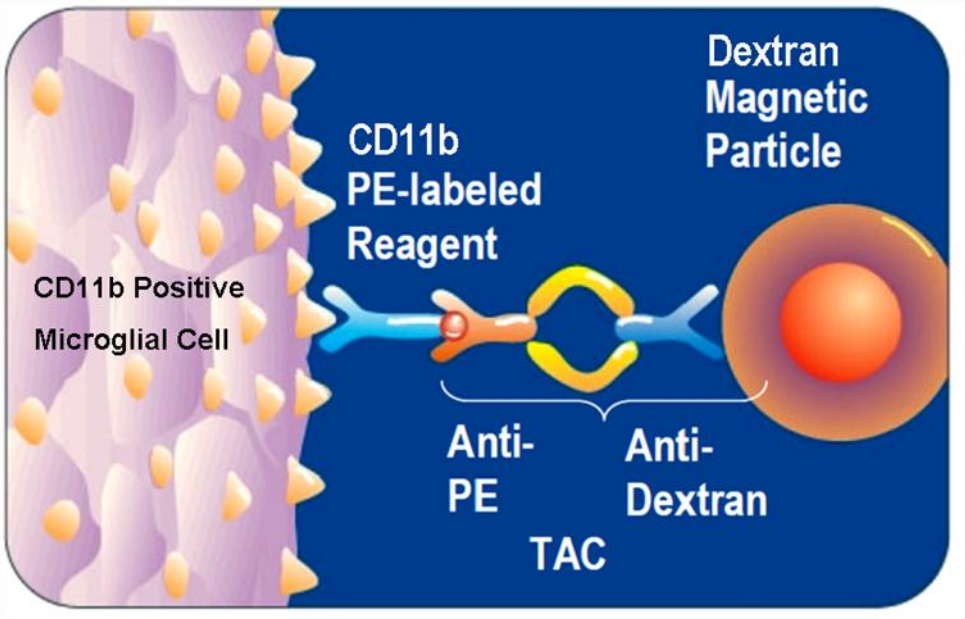



**Fig. 1. Principle of column-free magnetic separation.** Schematic illustration of CD11b-PE Tetrameric Antibody Complex (TAC) technology used for column-free immunomagnetic separation of microglia (A). Flow diagram (B) of steps involved in column free magnetic separation of microglia from postnatal mixed glial cultures (Graphics used with permission from STEMCELL Technologies Inc)

**Figure 2.**

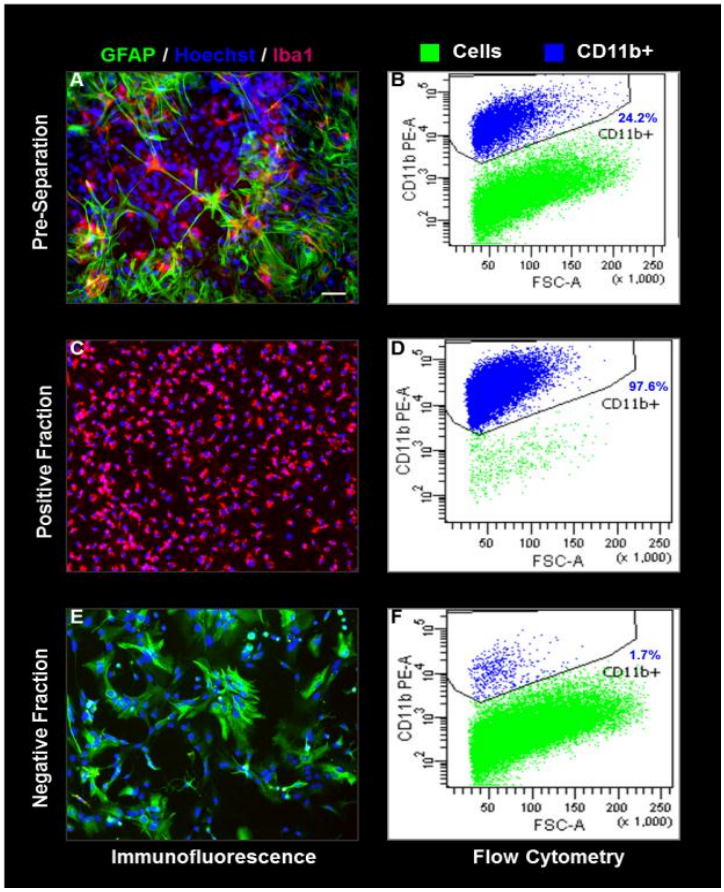
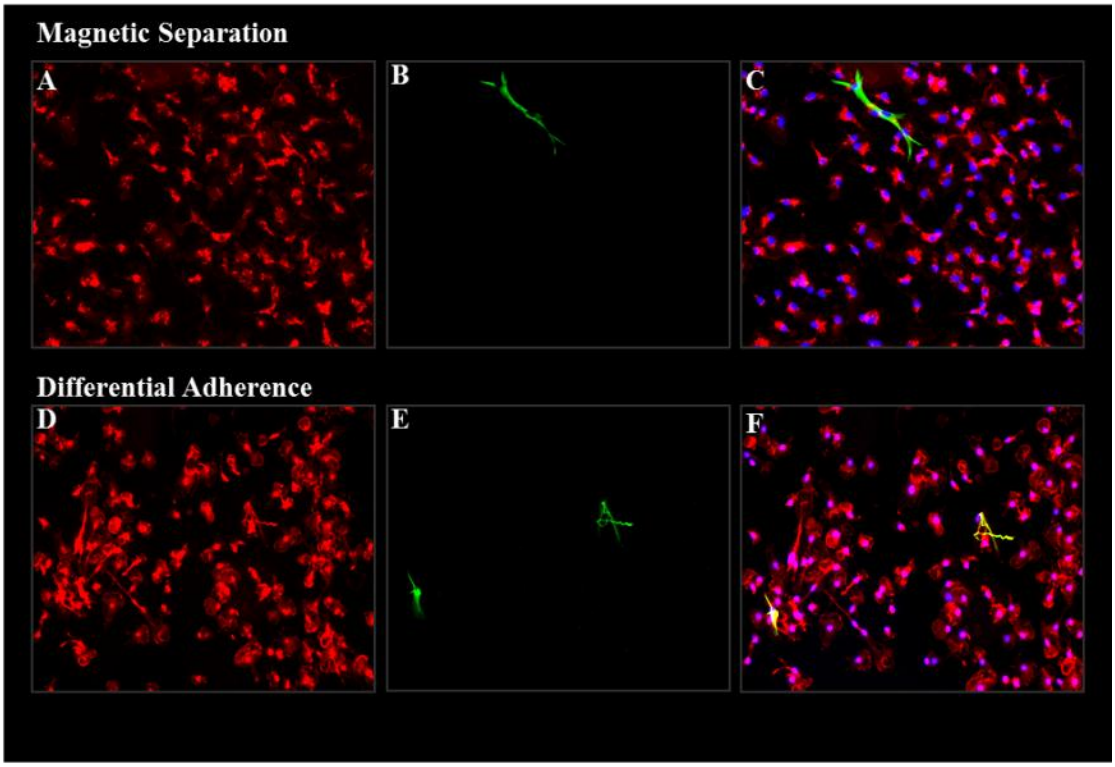



**Fig. 2. Analysis of microglial population before and after magnetic separation.** The proportion of microglia was determined quantitatively by flow cytometry with direct CD11b-PE labeling and also confirmed by iba1/GFAP/Hoechst immunofluorescence. Before separation, the mixed glial population contained around 26% $CD11b^+$ microglia (B,A). After magnetic separation, the positive fraction contained more than 97% pure microglia (D,C) while the negative fraction had less than 2% microglia with abundant GFAP-positive cells (F,E). Results from one representative separation are shown. Data for multiple separations is summarized in Table 1**.**

**Figure 3.**



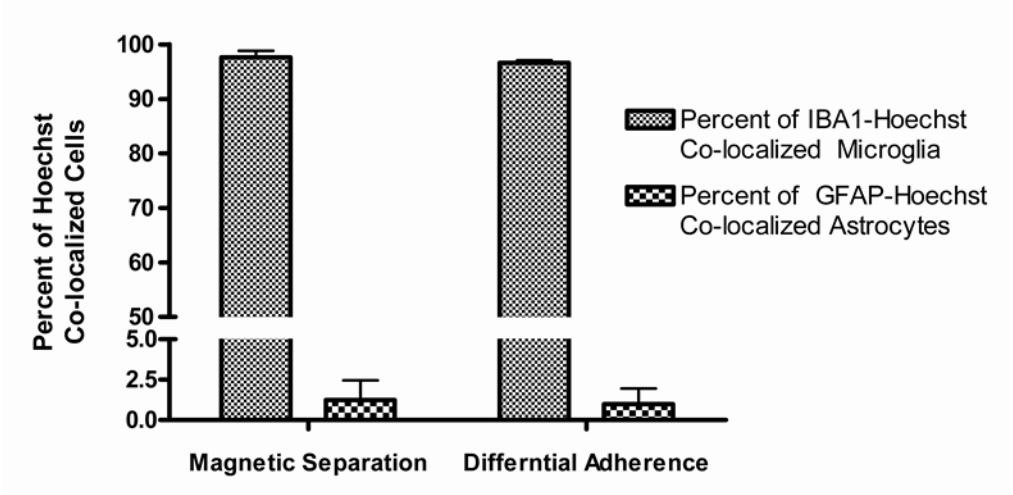




**Fig. 3. Comparison of microglial cell purity obtained by differential adherence and magnetic separation**. Triple labeling immunofluorescence was with iba1/GFAP/Hoechst

was used to compare microglial purity obtained by magnetic separation (A,B,C) and differential adherence (D,E,F). As shown above both methods had comparable purities with greater than 97% iba1-positive microglia. The number of Hoechst co-localized iba1-positive microglia and GFAP-positive astrocytes were also counted to quantitatively estimate the purity levels (G)

**Figure 4.**

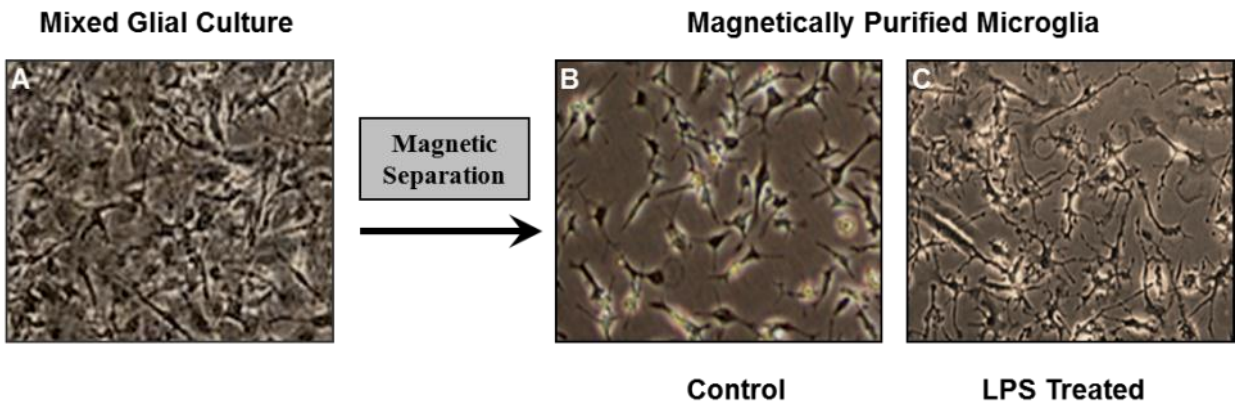



**Fig. 4. Morphological assessment of magnetically separated microglia.** Phase contrast microscopy was used to study microglial morphology. In confluent mixed glial cultures (A) abundant microglia are within the astrocyte layer. In magnetically separated microglia dramatic morphology changes, typical of activated microglia were observed with LPS (100 ng/ml for 24 h) treatment (C) compared to unstimulated microglia (B).

**Figure 5.**

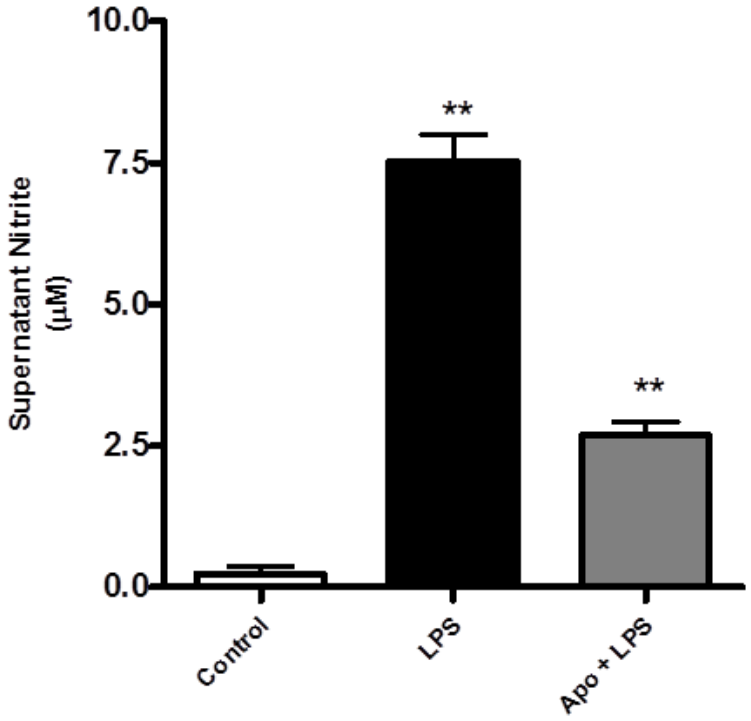



**Fig. 5. Nitrite levels in magnetically separated microglia.** Nitric oxide production was estimated by quantification of nitrite using the Griess reagent. LPS (100 ng/ml, 24 h) treatment induced a significant increase in nitrite production (** $p<0.01$) compared to control, which was attenuated by pretreatment with 100 µM apocynin (** $p<0.01$ compared to LPS).

**Figure 6.**

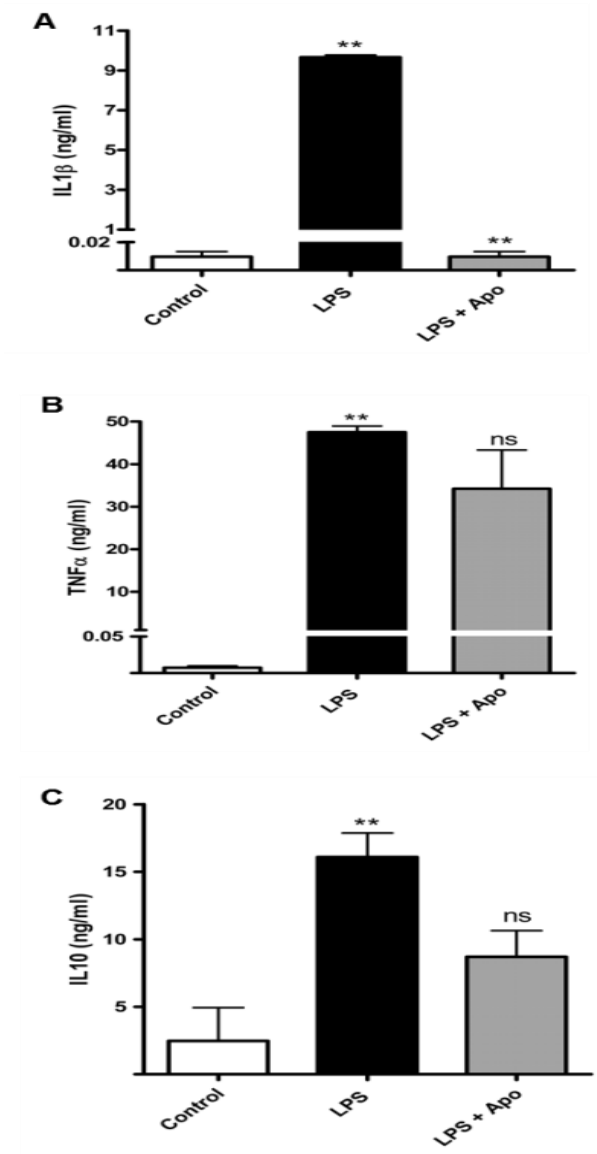



**Fig. 6. Cytokine release in magnetically separated microglia.** Supernatant cytokine levels were determined using a Luminex immunoassay system. LPS (100ng/ml, 24 h) treatment induced a significant increase in IL1β (A), TNFα (B) and IL-10 (C) production (** $p<0.01$) compared to control. Pretreatment with 100μM apocynin reduced cytokine release to different extents (** $p<0.01$ , ns - not significant)

**Figure 7.**

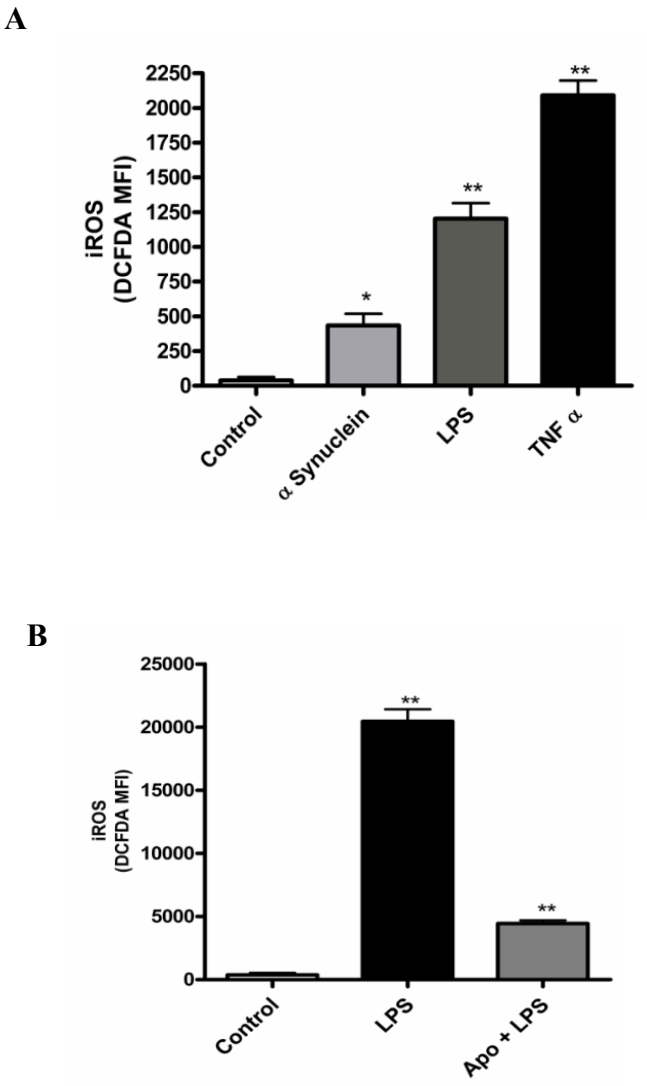



**Fig. 7. Intracellular ROS generation in magnetically separated microglia.** iROS was determined using the DCFH-DA probe. Stimulation with aggregated alpha synuclein (200nM), LPS (100ng/ml) and TNFα (30ng/ml) for 6 h induced significant iROS generation (* $p<0.05$, ** $p<0.01$) compared to control (A). Pretreatment with 100μM apocynin (B) significantly blocked LPS-induced iROS generation at 12 h (** $p<0.01$ ).

**Figure 8.**

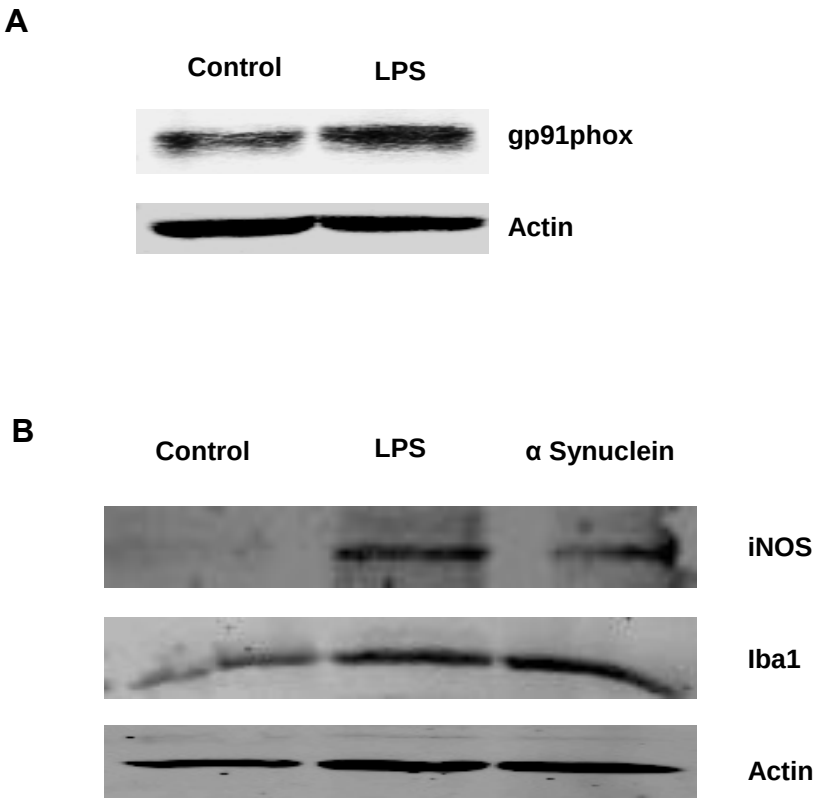



**Fig. 8**. **Western blot analysis of magnetically separated microglia.** Whole cell lysates from magnetically separated microglia were probed for iNOS, iba1 and gp91 phox protein levels after treatment. LPS (100ng/ml, 24 h) increased gp91 phox protein levels (A). Both LPS and alpha Synuclein (200nM) also upregulated iNOS and iba1 protein levels in magnetically separated microglia stimulated for 24 h.

**Table 1: Summary of flow cytometric estimates of CD11b$^+$ microglial population before and after column-free magnetic separation**

| Cell Population Analyzed | Percent of CD11b-positive cells determined by flow cytometry |
|---|---|
| **Total Mixed Glial Population** (Pre-separation) | **25.55 ± 3.2** |
| **Positive Fraction** (Magnetically Separated Microglia) | **97.65 ± 0.8** |
| **Negative Fraction** (Microglia Depleted Fraction) | **1.650 ± 0.3** |

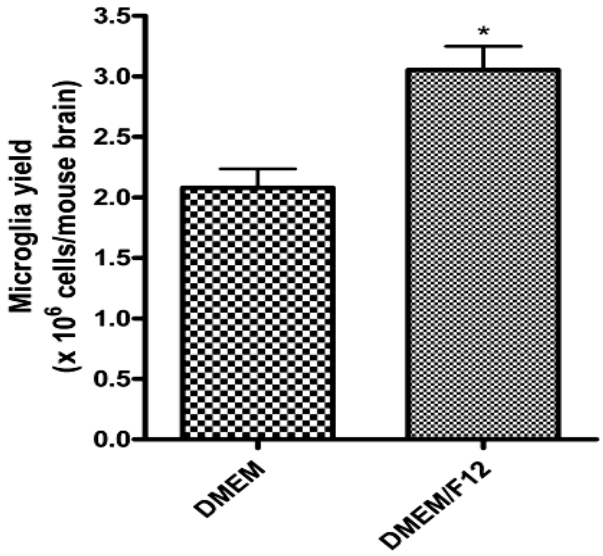
Data from 4 representative separations is expressed as the mean ± SE

**Table 2: Comparison of microglial yield and recovery with column-free magnetic separation and differential adherence methods**

| Microglia Separation Method | Yield Per Mouse Brain (x$10^6$ Cells) | CD11b$^+$ Cell Recovery | Purity |
|---|---|---|---|
| **Column-free Magnetic Separation** | **3.2 ± 0.38** | **87.98 ± 2.6%** | **>97 %** |
| **Differential Adherence** | **0.6 ± 0.18** | **25.94 ± 3.9%** | **>97 %** |

Data from 4 representative separations is expressed as the mean ± SE

**Supplementary Figure 1**




**Comparison of microglia yield from mixed glial cultures grown in DMEM and DMEM-F12 medium.** The yield of primary microglia per mouse brain obtained by magnetic separation from mixed glial cultures grown in DMEM or DMEM-F12 was determined by automated cell counting. Significantly higher yields of microglia (* $p<0.05$) were obtained with DMEM-F12 medium.

# CHAPTER V

## PROKINETICIN-2 IS SECRETED BY DOPAMINERGIC NEURONS FOLLOWING NEUROTOXICITY AND IS NEUROPROTECTIVE IN CELL CULTURE MODELS OF PARKINSON'S DISEASE

**A manuscript in preparation for The Journal of Neuroscience**

**Richard Gordon, Matthew L. Neal, Anamitra Ghosh, Vellareddy Anantharam, Arthi Kanthasamy and Anumantha G. Kanthasamy**

**Abstract**

Prokineticin-2 (PK2) is a recently discovered secreted protein that regulates diverse physiological functions in a tissue-specific manner. In the brain, PK2 signaling participates in olfactory bulb neurogenesis by directing the migration of subventricular zone progenitor cells and also controls circadian rhythms by functioning as an output molecule from the suprachiasmatic nucleus. PK2 expression however, is undetectable in other regions of the adult brain although its receptor PKR2 is ubiquitously expressed at high density. Herein, we demonstrate for the first time that PK2 is rapidly induced in nigral dopaminergic neurons in the MPTP model of Parkinson's disease and in dopaminergic N27 cells treated with $MPP^+$ or TNFα. We also report elevated levels of PK2 in nigral samples from postmortem PD patients demonstrating the clinical relevance of our findings. The PK2 GPCRs PKR1 and PKR2 are

both abundantly expressed in nigral tissue lysates, on primary dopaminergic neurons and N27 cells. TH-positive dopaminergic neurons in the substantia nigra expressed high levels of the PKR2 receptor. Our functional studies demonstrate that PK2 mobilizes calcium in N27 dopaminergic cells and protects against mitochondrial oxidative stress and neurotoxicity induced by $MPP^+$. We also demonstrate for the first time, that co-treatment with PK2 induced PTEN-inducible kinase-1(PINK1) in N27 cells to protect against $MPP^+$. We confirmed the neuroprotective functions of PK2 against $MPP^+$ using primary embryonic mesencephalon cultures. Together, our results identify a novel role for PK2 signaling during dopaminergic degeneration in PD. Our studies have implications for therapeutic targeting of the prokineticin GPCRs in PD and potentially other neurodegenerative diseases.

**Introduction**

Prokineticin-2 (PK2) is a recently discovered member of the AVIT protein family that has been shown to regulate diverse physiological processes including angiogenesis, hematopoiesis, reproductive functions, circadian rhythms and pain perception. In the CNS, PK2 regulates circadian rhythms by functioning as an output molecule from the SCN and is required for olfactory bulb biogenesis by directing migration of progenitor cells from the subventricular zone (Cheng et al., 2002; Cheng et al., 2005; Ng et al., 2005). The expression of PK2 however, has been shown to be largely restricted to the above regions, while its receptors are more ubiquitously expressed in the CNS, suggesting that PK-2 signaling could have distinct functions in other regions of the brain(Cheng et al., 2006).

While studying the gene expression changes induced by TNFα toxicity on dopaminergic N27 cells using PCR-array technology, we surprisingly found that the mRNA levels of PK2 were highly induced by TNFα. Since TNFα treatment is neurotoxic to N27 dopaminergic cells similar to $MPP^+$, we hypothesized that PK-2 could be a novel signaling mediator that is secreted following oxidative stress and neuronal injury. We tested this hypothesis with the MPTP animal model of PD and in vitro using MPP+ treated N27 cells. Western blot analysis and Immunohistochemistry studies confirmed that PK-2 was rapidly induced following MPTP treatment by dopaminergic neurons in the mouse substantia nigra in both C57 black mice and the GENSAT PK2-EGFP transgenic mouse model. In N27 cells, both $MPP^+$ and TNFα treatment increased PK2 expression and its release into the extracellular supernatant. We also confirmed that PK2 expression is elevated in the human PD brains corroborating the clinical relevance for PK2 signaling in PD.

PK2 has been shown to signal in both an autocrine and paracrine manner by activating two highly-similar G-protein coupled receptors, PKR1 and PKR2, both of which are expressed in distinct regions of the CNS (Lin et al., 2002; Soga et al., 2002; LeCouter et al., 2003; Shojaei et al., 2007). We characterized the expression of the prokineticin receptors in the nigrostriatal system and found that both receptors were expressed in nigral tissue lysates, primary dopaminergic neurons and N27 cells. Double-labeling immunohistochemistry revealed that dopaminergic neurons express PKR2, suggesting that PK2-PKR2 signaling could be functionally relevant during dopaminergic degeneration. Since PK2 is secreted following oxidative stress and neuronal injury by dopaminergic neurons and since its receptors are expressed by the same cells, we reasoned that PK2 signaling could

protect against dopaminergic degeneration. Functional studies revealed that PK2 can activate its receptors on dopaminergic neurons and co-treatment with recombinant PK2 protects against oxidative stress and neurotoxicity induced by $MPP^+$ in N27 cells and primary dopaminergic neurons. Mechanistic experiments into the signaling pathways by which PK2 protects against MPP toxicity showed that PK2-treated dopaminergic cells have increased levels of the PINK-1 protein which has been shown to protect dopaminergic cells against oxidative stress and mitochondrial dysfunction. Taken together, the results of our studies have profound implications for both PK-2 biology and the pathophysiology of PD. Given that the prokineticin GPCRs are prime drug targets and that PK-2 signaling is neruoprotective against dopaminergic degeneration, these results of this study have potential therapeutic applications to mitigate dopaminergic neuron loss in PD.

**Materials and methods**

**Chemicals and reagents**

DMEM/F-12, RPMI, fetal bovine serum, L-glutamine, IR-dye tagged secondary antibodies, Hoechst nuclear stain, penicillin, and streptomycin and other cell culture reagents were purchased from Invitrogen (Gaithersburg, MD). Recombinant TNFα was purchased from Peprotech. Antibodies for rabbit PKCδ, GRP-78 and iNOS were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse TH antibody was purchased from Chemicon (Temecula, CA) and rabbit Iba1 antibody was from Wako Chemicals (Richmond, VA). $^{32}$P-ATP was purchased from Perkin Elmer (Boston, MA) and the histone substrate from Sigma. The Bradford protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA).

RPMI, neurobasal medium, B27 supplement, fetal bovine serum, L-glutamine, IR-dye tagged secondary antibodies, penicillin, and streptomycin, MitoTracker dye and other cell culture reagents were purchased from Invitrogen (Gaithersburg, MD). Antibodies for Actin (1:2000), Tubulin (1:2000), Phospho-JNK (1:500) from Promega, Phospho p42/44 ERK1 (1:500) and Phospho p38 MAPK (1:500) from Cell Signalling, and PINK-1(1:500) was obtained from abcam (Cambridge, MA). Antibodies for rabbit and goat PK-2 (1:500), PKR1 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse TH antibody was purchased from Chemicon (Temecula, CA). Caspase assay substrates and inhibitors were purchased from MP Biomedicals (Solon, OH). The DNA fragmentation assay kit was purchased from Roche Applied Science. Bradford protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA). ROS generation determinant DCF-DA was purchased from Calbiochem(San Diego, CA). MPTP and recombinant rat TNFα was obtained from Sigma (St. Louis,MO). Recombinant murine TNF was from R&D Systems (Minneapolis, MN).

**Animal studies**

The C57/BL/6 mice were housed in a light-controlled (12 h light: 12 h dark cycle) and temperature-controlled mouse facility (22 ± 1°C) with food and water available at all times. Acute model MPTP injections, as previously described (Zhang et al., 2007b), were used with C57/BL/6 mice to determine levels of PK2 in the mouse brain. Intraperital (IP) injections into the body cavity of either saline or 18 mg/kg MPTP, dissolved in saline, were used to introduce the treatment once every 2 hours with a total of 4 injections. The mice were anesthetized with a mixture of ketamine-xylazine (100mg/kg, 10mg/kg), and perfused transcardially with 4% Paraformaldehyde (PFA) 24 hours after MPTP or saline treatment.

**PK2-EGFP transgenic mice**

The PK2-eGFP transgenic FVB/N-Swiss mice were generated at Rockefeller University by the GENSAT project using bacterial artificial chromosome (BAC) clone RP23-12A18, as described previously. The EGFP reporter cassette included a polyadenylation site that would prevent an overexpression of PK2 in the mice. The Mice were housed in a light-controlled (12 h light: 12 h dark cycle) and temperature-controlled mouse facility (22 ± 1°C) with food and water available at all times. Intraperital (IP) injections into the body cavity of either saline or 20 mg/kg MPTP, dissolved in saline, were used to introduce the treatment once every 2 hours with a total of 4 injections. The mice were anesthetized 24 hours after MPTP or saline treatment with a ketamine-xylaxine (100mg/kg, 10mg/kg) mixture, and perfused transcardially with 4% PFA. All animal procedures were approved by the Iowa State University Institutional Animal Care and Use Committee (IACUC).

**Cell cultures**

N27 rat dopaminergic neuronal cells, were grown in RPMI 1640 media supplemented with 10% Fetal Bovine Serum (FBS), penicillin (100 units/ml), and streptomycin (100 µg/ml), and 2 mM L-glutamine in 175mm$^3$ flasks as previously described (Clarkson et al., 1998; Kaul et al., 2003; Zhang et al., 2007b). N27 cells were kept in an incubator 37°C with 5% $CO_2$. Primary neurons were isolated from ventral mesencephalon tissue of gestational 14-day (E14) mouse embryos by dissection under a microscope. Neurons were collected, isolated and maintained according to our previously published protocols, using DMEM/F12

complete media (10% heat-inactive fetal bovine serum, 100 U/ml penicillin, 100µg/ml streptomycin Glutamate, 100µM non-essential amino acids, and 2mM Sodium Pyruvate)

**Western blotting**

Cells were collected after treatment, lysed using modified RIPA buffer, homogenized, sonicated, and centrifuged as previously described (Kaul *et al*., 2003; Kitazawa *et al*., 2002). The supernatants were collected and protein was normalized, then stored with loading buffer and DTT. The samples were run on a Sodium Dodecyl Sulfate (SDS) gel electrophoresis. The normalized protein samples were loaded into each well and first separated in a 4% stacking gel and then through a 12-18% resolution gel. Proteins were then transferred to a Nitrocellulose membrane and allowed to transfer overnight at 4°C fridge at 23-30 volts. After transfer the membranes were blocked using either Western blocking buffer (Rockland Immunochemicals) or with 5% nonfat dry milk powder. Membranes were washed several times using either PBS containing 0.05% Tween (PBST) or Tris buffer saline containing 0.05% Tween (TBST) wash buffers. Primary antibodies (in either blocking buffer or in a 5% BSA solution) were then added to the membranes and put at 4°C overnight. After another 4-5 wash steps, secondary antibody specific to the primary antibody is added (in either blocking buffer or in 5% lowfat dry milk solution) to the membrane for 1 hour. Either β-Actin or α-tubulin was used to confirm equal loading into each well of the gels. The membranes were washed 4-5 times with wash buffer, and then read using an Odyssey infra-red imager.

**SYBR Green qRT-PCR**

N27 cells were seeded out into $75mm^3$ flasks at $2 \times 10^6$ cells per flask. Treatments were performed in RPMI media supplemented with 2% FBS, penicillin (100 units/ml), and

streptomycin (100 μg/ml), and 2 mM L-glutamine. Cells were treated for 8 hours with $MPP^+$, or TNFα. After treatment, the cells were collected, pelleted, and resuspended in lysis buffer with β-Mercaptoethanol. RNA was isolated using an RNA isolation kit from Stratagene, Absolutely RNA Miniprep Kit. RT-PCR was performed using an AffinityScript qPCR cDNA synthesis system (Agilent Technologies) to convert the RNA into cDNA. Expression levels were determined using real-time PCR with Stratagene $RT^2$ SYBR green mastermix and prevalidated qPCR primers (SA Biosciences). Catalog number of the primer used was mouse PK2- PPM30250A. For normalization of each sample mouse 18SrRNA gene (catalog number-PPM57735E) was used as the housekeeping gene. The amount of each template was optimized with experiments to provide the maximum efficiency without inhibition of the PCR reaction. By the manufacturer's guidelines, dissociation curves and melting curves were run to ensure a single amplicon peak was obtained without any non-specific amplicons. The results are reported in fold change in gene expression, which was determined using the $\Delta\Delta C_t$ method using the threshold cycle ($C_t$) value from the housekeeping gene and for the respective gene of interest in each sample.

**Caspase and DNA fragmentation assays**

Enzymatic assays for caspase-3 activity were performed using acetyl-DEVD- amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC, 25 μm) as the fluorometric substrate for the reaction as described previously (Anantharam et al., 2002; Kaul et al., 2003). Synergy-2 Multi-Mode microplate reader was used to detect fluorescent signals generated upon cleavage of the AFC peptide substrate by caspase-3 at 510nm and excited at 400nm. Caspase activity was normalized by determining protein concentrations with the Bradford assay. For

DNA fragmentation assay, N27 cells were plated in 6-well plates at 0.8 x $10^6$ cells/well and treated the next day. The cells were pelleted and then gently lysed using the lysis buffer provided with the kit. The lysate was then spun down at 200 xg for 10 minutes and the supernatant was collected. The DNA fragmentation was quantified according to previously published methods (Anantharam et al., 2002; Kaul et al., 2003; Zhang et al., 2007b) using the Cell Death Detection ELISA Plus assay kit (Roche Applied Science). The plates were read by a Synergy-2 Multi-Mode Microplate Reader (BioTek Instruments, Inc) with absorbance at 405nm and an ABTS solution (reference wavelength at ~490) as a blank. The amount of protein in each lysate served to normalize the raw absorbance values.

**Glutathione assay**

For Glutathione assay, cells were seeded into a 25 $mm^3$ flasks at 1 x $10^6$ cells per flask in N27 growing media (RPMI with 10%FBS, penicillin (100 units/ml), and streptomycin (100 µg/ml), and 2 mM L-glutamine). The next day cells were treated in RPMI supplemented with 2% FBS, penicillin (100 units/ml), and streptomycin (100 µg/ml), and 2 mM L-glutamine. Collected the cells by scraping the flasks and pelleted the cells. The pellet was resuspended with PBS to wash off remaining media, and aliquoted into 1.5ml microcentrifuge tubes. Pelleted the cells again, and removed any PBS from the tube. Then the cells were lysed using 50mM Tris at pH 7.4, 5mM EDTA and 0.001% Butylated hydroxytoluene (BHT). The mixture was vortexed and kept on ice for 15 minutes, then vortexed again. Cells were then sonicated using a Fisher Sonic Dismembrator model 500. Then cells were then put into the centrifuge at high speed (13,000 RPM) for 10 minutes. Using 180µl of the resulting supernatant, added 22.5µl of 1mM Monochlorobimane in 50mM

Tris(pH 7.4), and 22.5µl Glutathione-S-transferase (10U/ml in 50mM Tris(pH 7.4) and was mixed well. Then transferred 200µl to a black-walled 96-well plate and incubated in a thermoshaker for 30 minutes at 23C. The plate was read at Excitation 380nm and Emission 470nm.

**Mitochondrial fragmentation analysis**

For Mitochondrial dysfunction experiments, cells were plated out onto dishes coated with 0.1% Poly-D-Lysine in RPMI with 10% FBS, penicillin (100 units/ml), and streptomycin (100 µg/ml), and 2 mM L-glutamine. Cells were treated in RPMI with 2% FBS, penicillin (100 units/ml), and streptomycin (100 µg/ml), and 2 mM L-glutamine for 16 hours. After treatment, the MitoTracker dye was added to each dish at a concentration of 1:2000µl from a stock of 1mM. The cells were incubated with the dye for 15-20 minutes at 37C. After incubation, cells were fixed with 4% paraformaldehyde (PFA) for 1 hour and then washed several times with PBS. Cells were then imaged using Nikon 2000U microscope, and all images were processed in MetaMorph 5.7 from Universal Imaging (Downingtown, PA).

**Detection of intracellular ROS generation in N27 cells**

To determine the level of intracellular reactive oxygen species (iROS) the fluorescent probe 2',7'-dichlorodi-hydrofluorescein diacetate (DCFH-DA) (Calbiochem). This chemical was used according to previously published reports (Zhang et al., 2005; Qian et al., 2007). Rat dompaminergic immortalized neuronal N27 cells were seeded into a 96-well plate. The N27 cells were treated with 200µM MPTP in RPMI media with 2% Fetal Bovine Serum penicillin (100 units/ml), and streptomycin (100 µg/ml), and 2 mM L-glutamine for 8 hours. The N27 cells were also cotreated with several doses of PK2 ranging from 5 nM up to 50 nM

along with the MPTP treatment. After the treatment, the cells were washed with warm HBSS media, and then incubated with 100µl of 40µM DCFH-DA in HBSS for 1 hour. The fluorescence intensity of the signal was determined using a Synergy-2 multi-mode microplate reader at an excitation of 485/20 and an emission of 530/25. The control fluorescent signal was treated as a control and subtracted as background. The fluorescence intensity increased in treatments was expressed as an increase in iROS as previously described (Zhang et al., 2005; Qian et al., 2007).

**Fluo-4 calcium mobilization assay**

To Fluo4 NW assay kit was used to test the calcium mobilization in N27 cells and primary neurons due to the effects of nanomolar concentrations of recombinant PK2. The cells were plated into a 96-well plate in their respective growth media. Cells were then washed with HBSS without calcium and magnesium. Then HBSS was added to each well along with the 1X Fluo4 NW dye (Molecular probes). The plate was then read kinetically using a Synergy-2 multi-mode microplate reader. After 20 seconds of reading the plate every 50 milliseconds for a background reading recombinant PK2 was added to the HBSS for final concentrations of 5nm, 10nm, 15nm, 20nm, or 25nm. The fluorescence was read every 50 milliseconds for 3 minutes. The net change in fluorescent signal ($D_t$) was obtained after subtracting the background from the maximum signal for each sample.

**Histology and immunocytochemistry.**

For immunocytochemisty, cells were plated onto coverslips in 24 well plates coated with 0.1% mg/ml poly-D-Lysine. After cells were treated, 4% formaldehyde was used to fix the cells for 30 minutes. The cells were washed 4-5 times with PBS wash buffer. Then block

buffer containing 2% BSA, 0.2% Triton X-100, and Tween was then added to the wells for 1 hour to permeabilize the cells. The cells were incubated with primary PK2 (Rabbit polyclonal, 1:500 dilution) and Tyrosine Hydroxylase (mouse monoclonal,1:2000) antibody in 2% BSA at 4C overnight. After 4-5 more wash steps an Alexa dye-conjugated secondary antibody in 2% BSA was added and incubated at room temperature on a shaker for 1 hour. Cells were then washed 4-5 times, then Hoechst counterstain was added for 15-20 minutes to label the nucleus. After 4-5 more washes the coverslips were mounted onto slides with Fluoromount mounting media. Cells were imaged under a Nikon 2000U microscope with a SPOT camera, and all images were processed in MetaMorph 5.7 from Universal Imaging (Downingtown, PA).

For histology, the mice were anesthetized using the ketamine-xylazine mixture and the brains were perfused with 4% PFA and then collected into 4% PFA and stored at 4°C for 48 hours. Brains were then removed, washed 3 times with PBS, and placed into a 30% sucrose solution for at least 24 hours. The brains were then put into Optimal Cutting Temperature (OCT) compound and frozen into blocks for cutting sections. Sections of the brain were cut using the Cryostat sectioning machine at -20°C and placed into Cryosolution (30% Sucrose, Ethylene glycol, and PBS). To perform antigen retrieval, the free-floating sections were heated for 30 minutes at 90°C in citrate buffer ( 10mM sodium citrate, pH 8.5). The sections were then washed with PBS and permeabilized with blocking buffer (2% BSA, 0.1% Triton X-100, and Tween) for 1 hour at room temperature. Antibodies directed to PK2 (Rabbit polyclonal, 1:500), PKR1 (Goat polyclonal, 1:1000), PKR2 (Rabbit polyclonal, 1:1000), Tyrosine Hydroxylase (Mouse monoclonal, 1:2000) were then incubated with the

sections overnight at 4°C. After several washes with PBS, the sections were incubated with Alexa dye-conjugated secondary antibodies for 1 hour at room temperature. After several more washed with PBS, Hoechst dye (1:3000) was added to the sections for 20 minutes at room temperature to stain the nucleus. The sections were then mounted on slides using prolong antifade gold mounting medium (Molecular probes) according to the manufacturer's instructions. A Nikon inverted fluorescent microscope (TE-2000U) was used to visualize the sections, and a spot digital camera was used to capture the images (Diagnostic Instruments Inc.).

**Data analysis**

Data analysis was performed using the Prism 4.0 software package (GraphPad Software, San Diego, CA). The data was first analyzed using one-way ANOVA and then Bonferroni's post-test was performed to compare all treatment groups. Differences of $p<0.05$ were considered statistically significant. The Student's t-test was used when differences between two groups were being compared.

**Results**

**Prokineticin-2 is highly induced in dopaminergic neuronal cells during $MPP^+$ and TNFα-induced cell death in vitro**

While studying the neurotoxic effect TNFα on dopaminergic N27 neuronal cells, we made a serendipitous discovery using PCR-array technology that PK-2 mRNA expression was highly induced early during TNFα induced cell death. Based on this unexpected observation, we hypothesized that PK-2 could be a novel secreted protein that is induced

early during dopaminergic neuron injury. To further validate our hypothesis and the relevance of our findings to PD, we tested if PK2 was induced in N27 cells during treatment with the Parkinsonian toxin $MPP^+$. N27 cells were treated with either TNFα (30 ng/ml) or a low dose of $MPP^+$ (200 μM) for 12 hours, and the level of PK2 was determined in cell lysates and cell culture supernatants. Both TNFα and MPP+ treatment resulted in increased PK2 protein expression in the N27 cells as determined by Western blotting. PK2 levels in the supernatant also increased concomitantly demonstrating that PK-2 is both upregulated and secreted by dopaminergic neuronal cells following inflammatory or oxidative stress signaling and neurotoxicity (Fig. 1A). We also visualized the expression and localization of PK2 using immunofluorescence in N27 cells. A rabbit polyclonal antibody that recognizes an epitope within the internal region of PK2 was used for immunofluorescence detection along with a species-specific Alexa-488 (green) conjugated secondary antibody. The expression of PK2 was mostly cytosolic and appeared to localize to the secretory pathway with prominent accumulation around the perinuclear zone and the ER network (Fig.1B). The localization pattern of PK2 in N27 cells is consistent with that of a secreted protein, which would be expected given that PK2 contains a signal peptide at its N-terminus targeting it for constitutive secretion (Li et al., 2001; LeCouter et al., 2003). The secreted PK2 protein has been shown to signal through two highly similar G protein-coupled receptors, PKR1 and PKR2 that are ubiquitously expressed in various tissues including the CNS (Lin et al., 2002; Masuda et al., 2002; Soga et al., 2002).

In the mouse brain, PKR2 was found to be highly expressed in most regions of the brain by neuronal cells while PKR1 has been shown to be expressed in only a few brain

regions (Cheng et al., 2006). Since the receptors for PK2 were likely to be expressed on dopaminergic neurons, we tested if N27 cells expressed PKR1 and PKR2. Western blotting of N27 cell lysates probed for PKR1 and PKR2 using a goat and rabbit polyclonal antibody respectively revealed that both receptors were expressed by N27 cells (Fig. 1C). The expression of PKR1 was less compared to PKR2 and appeared to go down with $MPP^+$ treatment.

Together, these results demonstrate that dopaminergic neurons secrete PK-2 and express receptors for the secreted protein. The activation of the prokineticin GPCRs by PK2 has been shown to mobilize calcium and activate downstream signaling pathways involving PI3K and p44/42 MAPK (Lin et al., 2002; Masuda et al., 2002; Soga et al., 2002; LeCouter et al., 2003). We tested the functional activation of the prokineticin receptors by PK-2 on N27 cells by measuring calcium mobilization using the recombinant active form of the PK-2 protein. The calcium flux in N27 cells was determined using the Fluo-4 NW assay system. PK-2 mobilized calcium in N27 cells in a dose dependent manner (Fig.1D) in the low nanomolar range similar to other cell types. Since both PKR1 and PKR2 are capable of being activated by PK-2, the contribution of each specific receptor in mediating the effects of PK-2 on dopaminergic cells is unclear at this time. Collectively, these results demonstrate for the first time that PK-2 is a novel signaling mediator that is highly induced and secreted from dopaminergic neurons following oxidative stress and neuronal injury. Further, they demonstrate that receptors for PK-2 are highly expressed on N27 dopaminergic cells and can be functionally activated by soluble recombinant PK-2 in vitro.

**PK2 is induced in substantia nigra dopaminergic neurons in the MPTP model of Parkinson's disease.**

To substantiate our in vitro results obtained with PK2 using the N27 dopaminergic cell model, we sought to determine the relevance of these findings in vivo. We utilized the acute MPTP model of PD to verify our hypothesis that PK2 expression is induced during dopaminergic neuron injury and secreted as a novel signaling mediator. The MPTP model is the most widely used and accepted model for PD in rodents and recapitulates many of the key pathological aspects of the disease including selective loss of dopaminergic neuron cell bodies in the SNpc, striatal terminal degeneration, dopamine depletion and motor deficits. Significantly, a strong neuroinflammatory response also occurs in this model with TNFα levels reported to peak at around 24 hours after MPTP dosing. We first examined if there was increased expression of the PK-2 protein in SNpc lysates obtained from MPTP-treated mice. We used an established acute MPTP dosing regimen was with 18mg/kg MPTP injected i.p. every two hours for a total of 4 doses. Control mice received saline and mice were sacrificed at the indicated time points. As shown in fig. 2A, there is a significant increase in PK-2 expression in the substantia nigra 24 hours after MPTP treatment compared to saline treated mice. Importantly, no PK-2 expression was observed in the striatum of either saline or MPTP treated mice indicating that the dopaminergic neuron cell bodies in the SNpc rather than their striatal terminals could be the source of PK-2 induction. We then determined the expression levels of PK-2 in the mouse SNpc at different time points (3, 12, 24 and 72hr) following MPTP treatment to verify if PK2 induction is an early response to dopaminergic neuron injury. As shown in fig 2B, PK-2 expression was significantly increased over saline treated

mice as early as 3hrs and remained elevated upto 3 days later, the last time point studied, suggesting that increased expression of PK-2 by dopaminergic neurons is an early and persistent signal in the SNpc following dopaminergic neuron insult. To verify that dopaminergic neurons in the SNpc were the specific cell type expressing PK-2, we used double immunofluorescence labeling in mouse midbrain SNpc sections for PK-2 and tyrosine hydroxylase (TH), a specific marker for dopaminergic neurons. As shown in fig.2C, there is greater than 98% co localization between TH and PK2 expressing cells as well as a remarkable increase in PK2 expression in the entire SNpc tract. Interestingly, a few cells that are not TH-positive but adjacent to dopaminergic neurons also express PK2 in the SNpc but these are less than 2% of the total PK2 expressing cells and their identity remains to be defined. In saline treated mice we observed little to no expression of PK-2 without any distinct cellular staining patterns, although in the western blot experiments there appears to be a low level of expression. This observation however is in good agreement with a previous published report by Cheng et al., which characterized the expression of PK-2 in the entire adult mouse brain by in situ mRNA hybridization and reported no significant expression in the SNpc although the prokineticin receptor PKR2 was expressed. Further, the recently completed GENSAT (Gene Expression Nervous System Atlas) project; a publicly available gene expression atlas of the adult mouse brain based on BAC-eGFP reporter transgenic mice also reports no detectable PK-2 expression in the midbrain SNpc region in the PK-2 eGFP transgenic mouse line. Together, our results demonstrate that PK-2 expression is highly induced in dopaminergic neurons in the SNpc following MPTP injury and as such represents

the first in vivo evidence suggesting a function for this protein in PD and neurodegeneration in general.

**Induction of PK2 and eGFP expression in the substantia nigra of the GENSAT EGFP transgenic mouse model**

To further validate and characterize the secretion of PK-2 by dopaminergic neurons in the MPTP model of PD, we used the GENSAT bacterial artificial chromosome PK-2 EGFP transgenic mouse model [Tg (Prok2-EGFP) FH64Gsat] specifically (Gong et al., 2003). The transgenic mouse generated as part of the GENSAT project, contains an EGFP reporter transgene and a polyadenlyation sequence introduced between the PK-2 promoter and the first coding exon of the PK-2 gene. The transcription of the EGFP reporter therefore is driven by activation of the PK2 promoter. Further, EGFP transcription is terminated at the polyadenylation site so that its expression occurs without any changes in native PK-2 expression. In a recent study (Zhang et al., 2009) these transgenic mice were used to reveal the projections of PK2-expressing neurons in the SCN to different target structures. Significantly, this study found no EGFP positive neurons or PK-2 mRNA expression anywhere in the midbrain in normal adult transgenic PK2-EGFP mice suggesting that PK-2 is not expressed or is undetectable in this region of the brain. In concordance with this study, our experiments with C57 black mice (Fig.1) demonstrate that the expression of PK-2 is low or absent in the substantia nigra of saline controls but is highly upregulated following MPTP-induced dopaminergic neuron injury. To substantiate these novel findings and visualize the expression of PK-2 in the subtantia nigra we used the MPTP model of PD to study the PK-2 EGFP transgenic. All mice used in the study were genotyped according to the donor's

recommended PCR protocol and confirmed to be transgenic based on the presence of the 600 bp strain-specific PCR product (Fig.3A). We further confirmed the veracity of the EGFP reporter for PK-2 in the transgenic mouse brain by visualizing EGFP expression in the olfactory bulb and the suprachiasmatic nucleus where PK-2 is constitutively expressed at high levels in the normal mouse brain. As expected, we observed extensive neuronal EGFP fluorescence in both the olfactory bulb and SCN (Fig. 3B) demonstrating that the expression of the transgenic EGFP reporter correlates accurately with the expression of PK-2 in the brain. The PK2-EGFP transgenic mice were treated with MPTP (4 x 20 mg/kg at 2 h intervals) and controls received equivalent volumes of saline. Nigral tissue was dissected 24 h later and lysates were probed for both EGFP and PK-2 protein levels by Western Blotting. We observed a substantial increase in the levels of the EGFP protein in MPTP-treated nigral tissue lysates along with a concomitant increase in the expression of PK-2. Actin was used as the internal control to demonstrate equal amount protein loading (Fig. 3C). Next, we imaged EGFP fluorescence directly in the substantia nigra using free floating tissue sections obtained from transgenic mice treated with saline and MPTP. The dopaminergic neurons in the substantia nigra were labeled with TH (red, Alexa 555) and EGFP/PK-2 expression was visualized directly by GFP fluorescence imaging (green). In nigral sections from saline treated mice, the amount of EGFP fluorescence was almost absent or barely above that of tissue autofluorescence (Fig. 3D). However, in MPTP-treated mice, intense EGFP fluorescence was evident along the nigral tract. Further, most of the EGFP-positive cells were also TH-positive indicating that nigral dopaminergic neurons produce the EGFP reporter for PK2 following MPTP treatment indicative of active PK-2 transcription in these

cells. Together, these results demonstrate that PK-2 is highly expressed in substantia nigra dopaminergic neurons of the PK2-EGFP transgenic mouse in the MPTP model of PD. They also demonstrate that the induction of PK-2 by dopaminergic neurons is not strain specific with similar results were also obtained in C57 black mice (Fig.1).

**PK2 is elevated in the substantia nigra of PD brains**

The primary pathological hallmark of Parkinson's disease involves a selective loss of melanized dopaminergic (DA) neurons in the substantia nigra compacta of the midbrain. Emerging studies suggest that PD is the outcome of complex pathological mechanisms involving environmental, genetic and cellular processes that result in dopaminergic cell death over time (Obeso et al., 2010). Although the precise etiologies involved are complex, oxidative stress and mitochondrial dysfunction have been identified as common downstream effector mechanisms that can drive dopaminergic degeneration in the SNc. Further, recent studies suggest that reactive microgliosis and the chronic neuroinflammatory response that occurs in the SNc can sustain dopaminergic degeneration and result in progressive neuronal loss (Block et al., 2007). Both clinical studies and animal models of PD have strongly implicated a role for TNFα signaling in modulating the neuroinflammatory responses in PD with elevated levels of TNF seen in human PD brains and all animal models of the disease (Wu et al., 2007; McCoy et al., 2008; De Lella Ezcurra et al., 2010; Harms et al., 2011). Our initial discovery that PK-2 is induced and secreted from dopaminergic neuronal cells in vitro following $MPP^+$ and TNFα treatment led us to believe that PK-2 could be a novel secreted factor induced early during oxidative or inflammatory stress in dopaminergic neurons. We then confirmed these findings in vivo using the MPTP model of PD in two different strains of

mice. Although the MPTP model does not recapitulate all the clinical or pathological features of idiopathic PD, oxidative stress and mitochondrial dysfunction are the primary mechanisms that account for the selective vulnerability of dopaminergic neurons observed in this model.

In order to establish the clinical relevance of PK-2 signaling in PD, we determined if elevated levels of PK-2 were also present in PD brains. SNc lysates from PD patients and age-matched controls were obtained from the University of Miami Brain Endowment Bank. The amount of PK-2 in the lysates was determined by Western blotting with 50 μg of total protein per sample. Actin was used as the internal control to demonstrate equal protein loading. Increased PK-2 expression was evident in most PD brains with low to undetectable levels in most age-matched controls (Fig 4A). We also observed some intrinsic variability in the PK-2 expression levels as would be expected with human clinical samples. To objectively quantify if PK-2 was elevated in PD brain lysates, we used densitometric analysis. The PK-2 band intensities from all six controls and PD samples were quantified using the Fujifilm Multi Gauge software and were normalized to those of actin. The normalized band intensities for the PD brain samples were then plotted as a fold-change over controls (Fig. 4B). As shown, PK-2 was increased almost 3-fold in the PD brain lysates compared to controls despite some intrinsic variability in expression levels. We also studied the localization of PK-2 in human PD brain sections using double-labeling fluorescence immunohistochemistry for PK-2 and TH. Nigral brain sections from postmortem PD patients and age-matched controls were processed for immunohistochemistry with a rabbit polyclonal antibody against PK-2 (green, Alexa-488) and a mouse monoclonal to TH (red, Alexa-555). Both primary antibodies detect the human epitope of the respective target protein. Species-specific Alexa-

dye conjugated secondary antibodies were used to visualize each protein and Hoechst stain was added to label the nucleus (blue). As shown in Fig. 4C, PK-2 staining was almost absent around the melanized TH-positive dopaminergic neurons in control sections where extensive TH staining is apparent. However in PD brain sections, intense and specific PK-2 staining was apparent and localized to the few surviving TH-positive dopaminergic neurons. PK-2 staining was also apparent in few cells (less than 5%) that were not TH-positive similar to the expression pattern for PK-2 observed in mice treated with MPTP (Fig. 1).

Together these results demonstrate for the first time that PK-2 expression is elevated in the SNc of PD patients and provides immense clinical relevance to our findings. Further, since PK-2 is a constitutively secreted protein, these findings suggest that PK-2 levels may be elevated in the CSF of PD patients. This could be particularly relevant for PK-2 as a potential PD biomarker since PK-2 appears to be induced and secreted as early as 3 hours after MPTP treatment in the mouse substantia nigra. More detailed time course studies would be ecessary to determine if the elevated levels of PK-2 correlate with onset and disease progression and if there are changes in CSF or serum PK-2 levels with the onset of PD.

**Receptors for secreted prokineticin-2 are expressed in the mouse substantia nigra and on primary dopaminergic neurons**

Having established that PK-2 is induced and secreted from dopaminergic neurons in vitro, in the MPTP mouse model of the disease and in postmortem PD patients, we sought to identify the target cells for the secreted protein in order to understand its potential functions. Two highly similar G protein coupled receptors for PK-2, have been identified and cloned by independent groups (Lin et al., 2002; Masuda et al., 2002; Soga et al., 2002). The two

GPCRs, PKR1 and PKR2 share around 85% amino acid identity between them although they are expressed on different chromosomes. Both PKR1 and PKR2 have are widely expressed in the gasterointestinal system, the reproductive organs and other peripheral tissues (Soga et al., 2002; LeCouter et al., 2003). Within the central nervous system the expression patterns of both receptors have been previously characterized. While PKR2 has been shown to be ubiquitously expressed throughout the brain, PKR1 appears to be restricted mostly to the olfactory region, dentate gyrus, subventricular zone and the dorsal motor vagal nucleus (Cheng et al., 2006). We determined the expression of prokineticin receptors in the mouse substantia nigra and in primary EVM neuronal cultures. Nigral tissue was dissected from C57 black mice treated with saline or MPTP (4 x 18 mg/kg) and lysates were prepared. Western blot analysis revealed that both prokineticin receptors were expressed in the substantia nigra tissue. Interestingly, the amount of PKR1 appeared to go down in MPTP-treated animals while that of PKR2 remained the same after treatment (Fig.5A). Primary microglia and astrocytes have been reported to express high levels of PKR1 compared to PKR2 (Koyama et al., 2006) which may account for the expression of PKR1 seen in nigral tissue lysates. We also confirmed the expression of both prokineticin receptors on primary microglia and astrocytes in our preliminary studies by Western blotting (data not shown). Our own studies with the N27 dopaminergic cell line revealed that the dopaminergic neurons themselves expressed both prokineticin receptors (Fig.1C) suggesting potential autocrine or paracrine functions for secreted PK-2 in the substantia nigra. We determined the expression of prokineticin receptors on dopaminergic neurons in the substantia nigra by double-labeling immunohistochemistry using TH as a marker for dopaminergic neurons. We found that TH-

positive dopaminergic neurons expressed high levels of PKR2 (Fig. 5B) which was evident as punctate staining around the periphery of the cells at high magnification. The secreted PK-2 from dopaminergic neurons that is induced following neuronal injury or oxidative stress therefore could potentially act on adjacent dopaminergic neurons expressing PKR2 to activate intracellular signaling pathways. We also tested the expression of prokineticin receptors on dopaminergic neurons in primary EVM cultures by double-labeling immunofluorescence. Primary EVM cultures were obtained from E14 timed-pregnant mice and grown in neurobasal medium to obtain pure neuronal cultures (>95% glial-cell free). Four-day old cultures were fixed and processed for immunocytochemistry with TH to label dopaminergic neurons and either PKR1 or PKR2. Hoechst was used to label the nucleus. PKR1 and PKR2 were expressed on all primary dopaminergic neurons as evidenced by colocalization with TH (Fig 5C). Both PKR1 and PKR2 were expressed by non-dopaminergic neurons as shown in the merged images with Hoechst staining. Together, these results confirm that the secreted PK-2 can act directly on dopaminergic neurons presumably via the PKR2 receptor which is expressed at high levels in vivo. In the light of previous reports showing high levels of PKR1 expression in microglia and astrocytes, our results here also suggest novel functions for secreted PK-2 in mediating neuron-glia communication following dopaminergic degeneration in PD. In this context, PK-2 that is secreted from dopaminergic neurons following neuronal injury could be a chemotactic factor for microglia and astrocytes via PK2-PKR1 signaling other than directly acting on adjacent dopaminergic neurons via PK2-PKR2 signaling pathways. Further, given the wealth of literature supporting a role of PK-2 in neurogenesis and as a cell survival factor, our finding that E-14

dopaminergic neurons express high levels of both PK-2 receptors implicate a potential role for PK-2 during neurogenesis and development and of the nigrostriatal system. The specific signaling pathways activated downstream of the PK-2 receptors in glial cells and dopaminergic neurons are currently under investigation in our laboratory.

**Soluble exogenous PK2 protects dopaminergic N27 cells from $MPP^+$ induced cell death and increases PINK1 protein levels.**

Having confirmed that the receptors for PK-2 being secreted from dopaminergic neurons are indeed expressed in the substantia nigra and that PKR2 is expressed on dopaminergic neurons themselves, we sought to determine the functional role of secreted PK-2. Within the brain, PK-2 signaling has been shown to regulate circadian rhythms by functioning as an output molecule from the SCN (Cheng et al., 2002; Cheng et al., 2005; Hu et al., 2007) and is also required for olfactory bulb biogenesis by acting as a chemoattractant for migration of progenitor cells from the subventricular zone (Ng et al., 2005; Prosser et al., 2007; Zhang et al., 2007a). Recently, PK-2 has been shown to cause excitability of the subfornical organ neurons and area postrema neurons and also depolarize parvocellular and magnocellular neurons further validating its role as a circadian output molecule.(Cottrell et al., 2004; Yuill et al., 2007; Ingves and Ferguson, 2010). However, unlike in the SCN and olfactory bulb where PK-2 is constitutively expressed, PK-2 is not detectable in the SN of normal mice but is upregulated in dopaminergic neurons following MPTP-induced oxidative stress and neuronal injury. The two receptors for PK-2 on the other hand are both expressed in the susbtantia nigra under normal conditions with PKR2 being present on dopaminergic neurons at high levels.

Since prokineticin has been strongly implicated in cell survival and angiogenesis (Kisliouk et al., 2005; Urayama et al., 2007; Li et al., 2010) and since PK-2 is secreted following dopaminergic degeneration, we hypothesized that soluble exogenous PK-2 could be a survival factor and protect against dopaminergic degeneration. We initially tested our hypothesis in N27 dopaminergic cells and determined if recombinant PK-2 could protect against oxidative stress and neurotoxicity induced by MPP+. N27 cells upregulate and secrete PK-2 in response to MPP+ similar to the nigral dopaminergic neurons in response to MPTP in vivo. Further, these cells express prokineticin receptors and mobilize calcium in response to exogenous PK-2 demonstrating that the receptors can be functionally activated by low nanomolar doses of the ligand (Fig.1-2). MPP+ toxicity on N27 dopaminergic cells has been well characterized with the primary mechanism of cell death being mitochondrial oxidative stress and activation of the intrinsic apoptotic cascade as a result of mitochondrial complex I inhibition. MPP+ treatment typically results in increased mitochondrial ROS generation, downstream activation of caspase-3, mitochondrial fission and DNA fragmentation. N27 cells were treated with 200 μM MPP+ and co-treated with recombinant PK-2 in 2% serum-containing medium. Total intracellular ROS levels were quantified using the DCFDA fluorescence assay. Caspase-3 activity was determined using a fluorimetric enzyme assay with a peptide substrate and DNA fragmentation was analyzed by a sensitive ELISA-based method. Co-treatment of N27 cells with PK-2 attenuated the generation of intracellular ROS by MPP+ in a dose-dependent manner at 8 h (Fig 6A). PK-2 treatment also reduced the activation of caspase-3 and DNA fragmentation induced by MPP+ treatment at 8 h and 16 h respectively (Fig. 6 B, C). We also qualitatively visualized mitochondrial fission

caused by MPP+ treatment using the fluorescent MitoTracker Red stain (Fig.6D). While MPP+ treatment alone caused evident fragmentation of mitochondria compared to untreated controls, cells cotreated with PK2 had intact mitochondria suggesting that PK-2 signaling counteracts the toxic effect of $MPP^+$ in these cells.

Taken together, these results demonstrate for the first time that PK-2 signaling can protect dopaminergic cells against $MPP^+$ mediated oxidative stress, mitochondrial dysfunction and cell death. They also suggest that the secreted PK2 from dopaminergic neurons could be a protective factor that acts locally in the substantia nigra to protect against acute oxidative stress and neurontoxic insults. The protective effect of PK2 treatment was also evident in the activation of pro-apoptotic signaling pathways in N27 with $MPP^+$ treatment. The early stages of mitochondrial membrane permeabilzation induced by $MPP^+$ is typically associated with activation of pro-apoptotic signaling pathways including ERK, JNK, MAP kinases and increased levels of pro-apoptotic bcl2 family proteins (Galluzzi et al., 2009). $MPP^+$ treatment resulted in increased levels of active JNK, ERK1/2 and the pro-apoptotic short form of MCL-1 ($MCL1_s$) all of which were reduced in cells co-treated with 10 and 25 nM of recombinant PK2 (Fig. 6E). Since the activation of JNK and ERK are typically associated with the early stages of pro-apoptotic signaling following mitochondrial membrane permeabilization, it is likely that PK2 exerts its protective effect by modulating early protective signaling pathways or increasing antioxidant defense mechanisms within the cell to protect against mitochondrial oxidative stress.

Having established that exogenous recombinant PK2 can protect against $MPP^+$ neurotoxicity in N27 cells, we sought to determine potential signaling pathways involved.

Since oxidative stress is a primary mechanism by which $MPP^+$ cell death proceeds and because co-treatment with PK-2 significantly reduced total intracellular ROS levels in these cells, we determined if PK-2 modulates antioxidant defense mechanisms or other pro-survival signaling pathways associated with mitochondrial integrity. PINK1 (PTEN-induced putative kinase 1) is a well-studied mitochondrial serine/threonine kinase that has been associated with mitochondrial integrity and pathology. Mutations in PINK-1 that result in a loss of function of the kinase have been linked to autosomal recessive Parkinsonism. Further, a loss of PINK-1 by either germline deletion or siRNA knockdown leads to significant functional impairment of the mitochondria with increased mitochondrial fragmentation and susceptibility to oxidative stress. Since co-treatment of N27 dopaminergic cells with PK-2 attenuated MPP+ induced intracellular ROS generation and mitochondrial fragmentation, both of which are dependent on PINK-1 signaling, we determined if PK-2 treatment affected the levels of PINK-1 within these cells by Western blot analysis. To our surprise, we found that the 55 kDa processed form of the PINK-1 protein (Fig. 6F) was increased in PK-2 treated cells that were being protected from MPP+ toxicity (Fig. 6). Mutation in the parkin gene have also been shown to have almost identical effects on mitochondrial morphology and function as PINK-1 and parkin could functionally compensate for the loss of PINK-1 suggesting that the two proteins operate in a common or overlapping genetic pathway. However, unlike PINK-1, the levels of parkin did not change with PK-2 treatment in the same lysates indicating that PK-2 signaling modulates the levels of PINK-1 but not parkin to protect against MPP+ induced oxidative stress and mitochondrial dysfunction. Taken together, our results here demonstrate for the first time that PK-2 signaling can protect

dopaminergic cells from oxidative stress, mitochondrial dysfunction and cell death induced by MPP+. More importantly, our results identify a novel link between PK-2 signaling and increased PINK-1 protein levels as a major compensatory response that mediates protection against MPP+ neurotoxicity in dopaminergic cells. These findings have immense implications for therapeutic targeting of the prokineticin receptors to prolong cell survival of dopaminergic neurons in PD by protecting against oxidative stress.

**PK2 protects primary dopaminergic neurons from $MPP^+$ induced degeneration**

Previous reports have demonstrated that PK-2 can act as a trophic factor for primary cells including cardiomyoctes and hippocampal neurons either by enhancing cell survival or by protecting against cell death induced by neurotoxic agents. To extend the results of our neruoprotective studies with PK-2 in dopaminergic N27 cells to a more relevant model system, we tested if soluble PK-2 could protect primary dopaminergic neurons from MPP+ induced neurotoxicity. Having first confirmed that both prokineticin receptors, PKR1 and PKR2, were expressed on primary dopaminergic neurons in culture (Fig. 4C), we validated the functional activation of the receptors by verifying the calcium flux generated by PK-2 in primary cultures using the Fluo-4 NW assay system. As shown (Fig. 7A), nanomolar amounts of PK-2 generated a dose-dependent calcium flux in primary neuron cultures. For neuroprotection studies, primary EVM cultures obtained from E14 timed-pregnant mice were grown in neurobasal medium and treated 4 days after plating. Cultures were routinely verified to be >95% glial-cell free at the time of treatment. Primary neuronal cultures at DIV-4 were treated with 5 μM $MPP^+$ for 24 h or co-treated with 25 nM PK-2. For the co-treatment group, PK2 was added again 12 h later. Dopaminergic neurotoxicity was quantified by the

dopamine uptake assay and TH-positive neuron counting. Dopamine uptake was reduced following $MPP^+$ treatment to almost 30% of the control levels at 24 h indicative of extensive dopaminergic degeneration. In cells co-treated with PK-2, dopamine uptake was significantly (#, $p<0.05$ vs $MPP^+$ alone) improved at around 50% of control cultures (Fig.7B). Similar results were obtained with TH+ neuron counts with PK-2 co-treated cultures having significantly (##, $p<0.01$ vs MPP+ alone) higher numbers of viable dopaminergic neurons compared to cultures treated with $MPP^+$ alone (Fig. 7C). The morphology of dopaminergic neurons in PK2 co-treated cultures as revealed by TH-immunofluorescence also displayed less degeneration with increased processes compared to cultures treated with $MPP^+$ alone. Together, these results demonstrate that PK-2 can protect primary dopaminergic neurons from $MPP^+$ toxicity. Further, they suggest that the secreted PK-2 from dopaminergic neurons can function as a local survival factor to protect against acute oxidative stress and neurotoxic insults.

**Discussion**

This report describes a novel role for prokineticin-2 signaling during dopaminergic degeneration with clinical relevance to the pathophysiology of Parkinson's disease. Herein, we demonstrate for the first time that PK2 is induced following dopaminergic degeneration and is highly expressed by dopaminergic neurons in cell culture and animal models of PD. We also confirmed that PK-2 is elevated in nigral tissue lysates of postmortem PD patients compared to age-matched controls, establishing the clinical relevance of our findings. We show that receptors for the secreted PK2 ligand are expressed on dopaminergic neurons in

the substantia nigra, in primary mesencephalic cultures and N27 cells and also that recombinant PK2 can functionally activate its receptors to mobilize calcium in dopaminergic neurons. Significantly, our functional studies demonstrate that PK-2 signaling can protect dopaminergic cells from $MPP^+$ induced oxidative stress and neurotoxicity by a PINK-1 dependent pathway.

The basis for these studies was an unexpected discovery that the prokineticin-2 mRNA is induced by more than 7-fold in N27 dopaminergic cells following exposure TNFα. Since TNF treatment is associated with dopaminergic neurotoxicity similar to $MPP^+$ in this model system, we hypothesized that PK2 could be a novel secreted neuropeptide that is released from dopaminergic neurons following neurotoxicity insult and oxidative stress. Indeed, both TNF and MPP+ increased the synthesis of PK-2 in N27 cells and its release into the culture supernatant, therefore confirming the validity of our hypothesis in vitro. We used the MPTP mouse model of PD to further investigate PK2 signaling in the nigrostriatal system since this model recapitulates many of the important pathophysiological features of PD, particularly with respect to nigral dopaminergic degeneration and oxidative stress (Jakowec and Petzinger, 2004; Fox and Brotchie, 2010). We demonstrate that PK2 was highly expressed as early as 3 h after MPTP treatment in substantia nigra tissue lysates but not in the striatum. Time course experiments revealed that increased PK2 expression was evident upto 3 days after MPTP treatment in the substantia nigra. Double labeling immunohistochemistry studies reveal that PK2 is specifically upregulated in TH-positive dopaminergic neurons of the substantia nigra. It must be noted that PK2 expression in saline-treated control mice was almost undetectable in nigral tissue lysates and brain sections suggesting that PK2 is not

expressed in this region of the adult brain under normal conditions but is induced following neuronal injury. This is in agreement with two previous reports that characterized the expression of PK-2 across the entire adult mouse brain and found no expression in regions of the midbrain (Cheng et al., 2006; Zhang et al., 2009). We also obtained identical results with the GENSAT PK-2 EGFP transgenic mouse model [Tg (Prok2-EGFP) FH64Gsat] where PK-2 expression could be traced using the fluorescent EGFP reporter protein. Since these transgenic white mice are on a Swiss Webster genetic background, which is distinct from that of the commonly used C57bl6 strain, the reproducibility of our results in this strain of mice further support the validity of our findings.

The transcriptional mechanisms by which PK2 induction occurs in dopaminergic neurons remain to be determined and would also be of therapeutic value given that our mechanistic studies suggest that PK2 has neuroprotective functions in vitro. The PK2 promoter has been shown to be replete with multiple E-box sequences (CACGTG) that are binding sites for by basic helix-loop-helix (bHLH) transcription factors such as HIF1α which has been shown to regulate the expression of PK-2 during angiogenesis (LeCouter et al., 2003). Previous studies have shown that the expression of PK-2 in the CNS is regulated by the CLOCK/BMAL1 in SCN in a circadian fashion and by Ngn1/MASH1 during olfactory bulb biogenesis. We posit that the induction of PK2 in dopaminergic neurons would most likely be under the control of HIF1α, which has been shown to be activated by oxidative stress and by TNFα under normoxic conditions (Jiang et al., 2010). Further, HIF1α activation has been reported in nigral dopaminergic neurons following MPTP treatment and also in N27 cells treated with MPP+, making it a likely candidate for transcriptional regulation of PK-2 in

this system (Sharp and Bernaudin, 2004; Lee et al., 2009; Correia and Moreira, 2010). The detailed transcriptional mechanisms regulating the induction of PK2 in dopaminergic neurons are currently under study in our laboratory.

Our data also demonstrate that both receptors for PK2, PKR1 and PKR2 are present in the substantia nigra with PKR2 being expressed on nigral dopaminergic neurons. While PKR1 could be detected in nigral tissue lysates in Western blots, we failed to detect its expression on nigral dopaminergic neurons by immunohistochemistry. Microglia and astrocytes have been shown to express high levels of PKR1 (Koyama et al., 2006) and it is possible that these are the primary PKR1-expressing cells in nigral tissue lysates. We also detected expression of both PKR1 and PKR2 on N27 dopaminergic cells and primary dopaminergic neurons in EVM cultures. Previous reports have shown that PKR2 is the dominant prokineticin receptor present in the brain and that a PK2-PKR2 signaling axis mediates the known functions of PK2 such as olfactory bulb biogenesis and regulation of circadian rhythms (Cheng et al., 2006; Zhou, 2006). It is likely that the same ligand-receptor combination is relevant to PK-2 signaling in nigral dopaminergic neurons.

A wealth of evidence supports a role for prokineticin signaling in cell survival in various tissues and PK2 is a known mediator of angiogenesis, hematopoiesis and neurogenesis(LeCouter et al., 2003; Battersby et al., 2004; LeCouter et al., 2004; Kisliouk et al., 2005). In a recent study, PK2 signaling was also shown to protect cardiomyocytes against oxidative stress and promote cardiomyocyte survival by activating the Akt pathway (Urayama et al., 2007). Since PK2 is upregulated and secreted in response to oxidative stress and neurotoxicity by dopaminergic neurons and since its receptors are expressed on the same

cells in vivo, we reasoned that the secreted PK2 could function as a neuroprotective factor and activate intracellular signaling pathways that mediate oxidative stress resistance and survival of dopaminergic neurons. We tested this hypothesis in N27 dopaminergic cells and primary neuron cultures and demonstrate for the first time that co-treatment with PK2 attenuates the generation of intracellular ROS by $MPP^+$ in dopaminergic cells. Further, PK2 treated cells were protected against downstream caspase-3 activation and DNA fragmentation by MPP+ providing clear evidence for activation of anti-apoptotic survival signaling by PK-2. The mechanism of $MPP^+$ toxicity in dopaminergic cells is primarily through the inhibition of complex I (NADH/ubiquinone oxidoreductase) of the mitochondrial electron transport chain and usually results in activation of the intrinsic apoptotic pathway and is accompanied by mitochondrial fission. In PK2 co-treated cells, the activation of pro-apoptotic kinases JNK and ERK were both attenuated compared to cells treated with $MPP^+$ alone. Further, the mitochondrial fragmentation that occurred 16 h after MPP+ exposure was significantly reduced in PK-2 treated cells where an intact mitochondrial network was evident at the same time point. The dopaminergic neruoprotective effect of PK2 against $MPP^+$ toxicity was also evident in primary mesencephalic neuronal cultures in which co-treatement with PK2 resulted in higher TH-positive neuron counts and increased uptake of tritiated dopamine compared to cultures treated with MPP+ alone. The protective effect of PK-2 on primary dopaminergic neurons against MPP+ toxicity was also evident morphologically when visualized using TH immunofluorescence.

To further characterize the mechanisms by which PK-2 protects dopaminergic cells against MPP+ toxicity, we looked at various protective signaling pathways relevant to

oxidative stress and mitochondrial dysfunction. In an unexpected finding, we noticed increased levels of the anti-apoptotic PINK-1 protein in N27 cells co-treated with PK2 and $MPP^+$ compared to cells treated with either $MPP^+$ alone or untreated controls suggesting that PK2 signaling can protect dopaminergic cells by a PINK-1 dependent mechanism. In recent years, the role of PINK-1 in protecting cells against oxidative stress and maintaining mitochondrial integrity has been well established (Clark et al., 2006; Gautier et al., 2008). Of particular importance to our results here, are studies which have demonstrated that cells deficient in PINK-1 have increased susceptibility to oxidative stress and mitochondrial dysfunction accompanied by increased fragmentation of the mitochondria (Lutz et al., 2009). On the other hand, overexpression of PINK-1 has been shown to protect cells against oxidative stress indicating that the expression levels of this kinase could be critical in determining the susceptibility or resistance of cells to mitochondrial dysfunction and oxidative stress (Petit et al., 2005; Mai et al., 2010). Our results demonstrating that PK2-treated cells are protected against $MPP^+$ induced oxidative stress and mitochondrial fragmentation are well explained by the fact that these cells have increased expression of increased PINK-1 expression and identify a previously unknown link between PK2 and PINK-1 signaling. Interestingly, the levels of Parkin, which has been shown to function analogous to PINK-1, did not change with PK2 treatment. Although further studies would be required to elucidate the detailed mechanism by which PK-2 increased PINK-1 levels in N27 cells, these finding suggests that there could be immense therapeutic value to developing PK2 mimetics as prokineticin GPCR agonists to increase the survival of dopaminergic neurons in PD

Taken together, the results of our studies presented here, have far-reaching implications for dopaminergic degeneration in PD and for PK2 biology in general. To our knowledge, this is the first report showing that PK2 can be rapidly induced following neurotoxic insults or neurodegeneration in the adult mammalian brain. Given that the prokineticin receptors, are ubiquitously expressed in the brain it is likely that the upregulation and release of PK-2 from neurons could be an early response to neuronal injury in different areas of the brain and could be associated with other neurodegenerative besides PD.

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**Figure 1.**

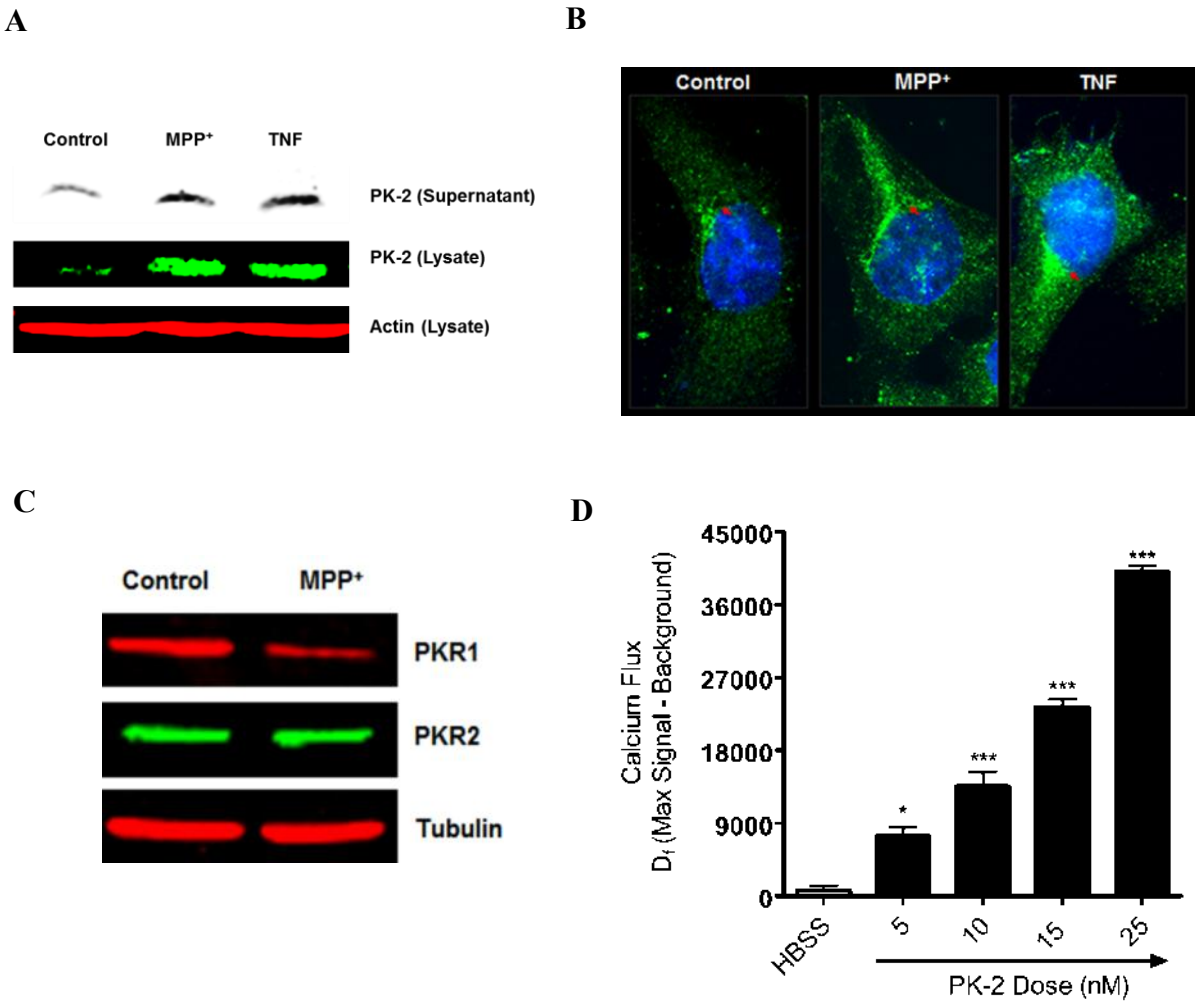



**Figure 1. Induction and release of PK-2 in dopaminergic N27 cells and calcium mobilization by recombinant PK-2. A,** Western blots for intracellular and secreted PK2 protein by N27 cells. N27 cells were treated with TNF (30 ng/ml) and $MPP^+$ (200 μM) for 12 h. At the end of the treatment, supernatants were collected and concentrated using a Vivaspin centrifugal concentrator with a 3 kDa molecular weight cutoff. Cell lysates were prepared using a modified RIPA buffer. Equal amounts of protein (50 μg/well) were loaded and samples were immunoblotted for PK-2 using a rabbit polyclonal antibody. Actin was

used as the internal control to verify equal protein loading. PK-2 expression was induced in N27 cells following exposure to TNF and $MPP^+$ in both cells and the extracellular supernatants indicating that these cells upregulate and secrete PK-2 similar to dopaminergic neurons in vivo. **B,** PK2 immunofluorescence in N27 cells. Intracellular localization of PK2 was determined using immunocytochemistry following exposure to TNF and $MPP^+$ for 12 h. Cells were incubated overnight in a PK2 primary antibody and visualized using a species-specific Alexa-488 (green) secondary antibody. Hoechst dye was used to stain the nucleus (blue). Images were captured using a Nikon TE-2000 fluorescence microscope at 60X magnification. Cytosolic expression of PK-2 (green) was evident and appeared to be localized in the secretory pathway. **C,** Western blot of prokineticin receptors PKR1 and PKR2 in N27 cells. Lysates were probed for PKR1 and PKR2 expression by Western blotting using a goat polyclonal antibody to PKR1 (red) and a rabbit polyclonal antibody to PKR2. Bands were visualized using a IR-dye tagged secondary antibodies. Tubulin was used as the internal control to demonstrate equal protein loading. Both PKR1 and PKR2 were expressed by N27 dopaminergic cells. **D,** Calcium mobilization by PK2 in N27 cells. Calcium flux induced by recombinant PK-2 in N27 cells was determined using the Fluo-4 NW assay kit with nanomolar doses of the recombinant PK-2 protein. Calcium-free HBSS was used on the control group. The calcium-flux data was expressed as the net change in fluorescence signal (Df) representing the maximum signal after background subtraction for each dose of PK-2. As shown, PK2 mobilized calcium downstream of its two GPCRs in a dose-dependent manner in N27 cells. Asterisks denote a significant (* $p<0.05$ and *** $p<0.001$) difference between control and PK-2 treated groups.

**Figure 2.**

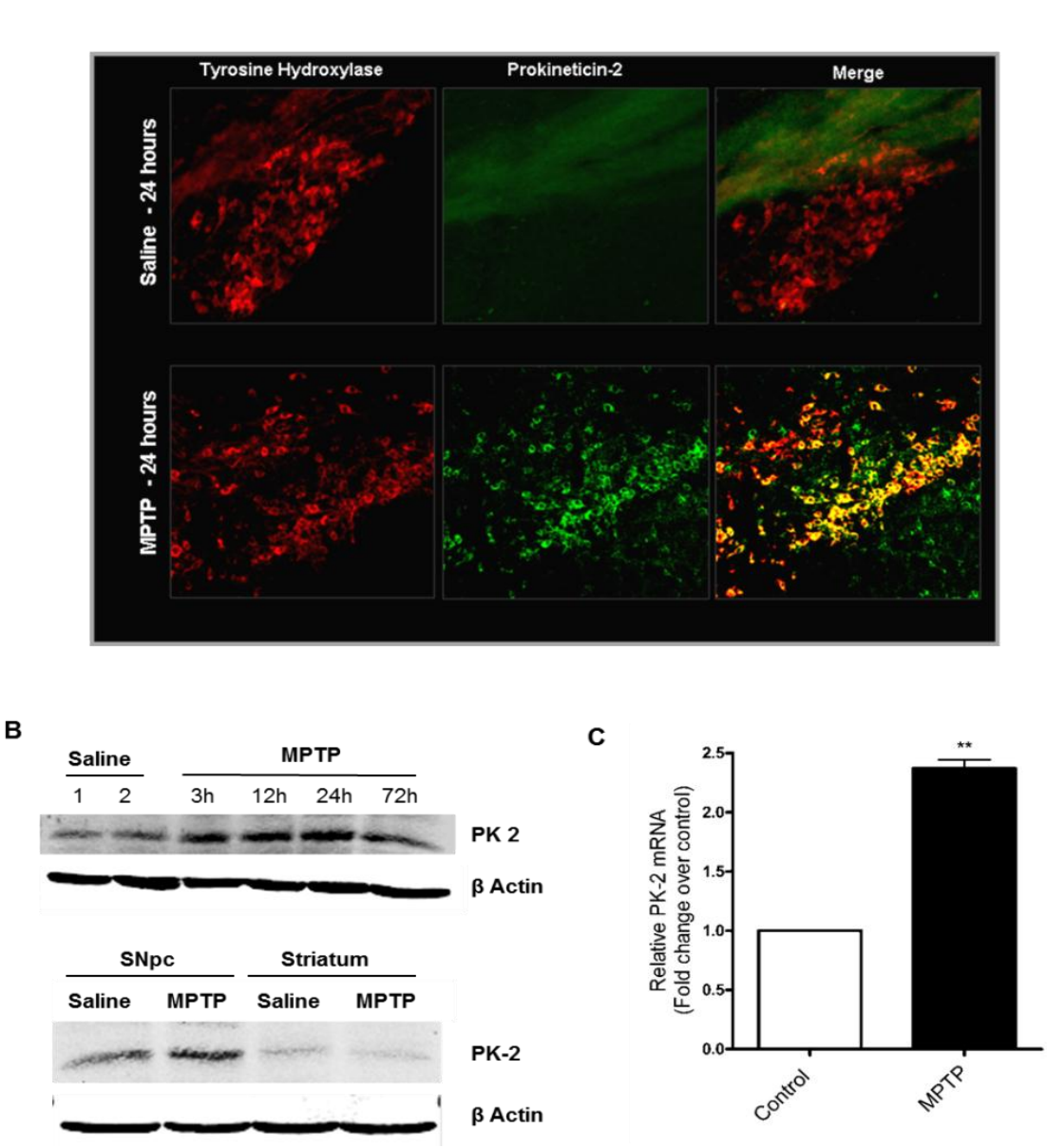



**Figure 2. Prokineticin-2 is highly induced in substantia nigra dopaminergic neurons following MPTP treatment in a mouse model of Parkinson's disease.** C57bl/6 mice were injected with 4 doses of MPTP (18 mg/kg) at 2 h intervals. Mice were sacrificed at various time points after the last injection. **A, Double** labeling immunohistochemistry for PK-2 and

TH in mouse substantia nigra sections. Mice were anesthetized and perfused transcardially with 4% PFA 24 h after MPTP treatment and 30 micron sections were cut using a freezing microtome. Substantia nigra sections were stained for TH (red - Alexa 555) and PK-2 (green - Alexa 488). High levels of PK-2 expression was evident in almost all TH-positive neurons as seen in the merged image (yellow color). A few adjacent cells with neuronal morphology that were not positive for TH also displayed PK-2 in the nigral region. Representative images at 20X magnification are shown. **B,** Western blots for PK-2 protein expression. Increased nigral PK-2 expression was also confirmed by Western blotting. Mice were sacrificed at 3 , 12 , 24 and 72 hours after MPTP treatment. Nigral and striatal tissues were dissected out using a brain matrix and lysates were probed for PK-2 by Western blotting with a rabbit polyclonal antibody. Increased PK-2 expression in the substantia nigra (top panel) was evident as early as 3 h after the last MPTP injection and remained elevated upto 72 hours later. Interestingly, while PK-2 expression was increased in the substantia nigra lysates at 24 h (bottom panel), its level in the striatum remained the same after MPTP treatment. Representative blots are shown and experiments were repeated more than three times **C**, Realtime qRT-PCR for PK-2 mRNA expression in the substantia nigra. PK-2 mRNA expression in substantia nigra tissues was determined 24 h after MPTP treatment by real-time SYBR green qRT-PCR using pre-validated primers. 18S rRNA was used as the internal standard for normalization and the fold increase in gene expression was determined by the $\Delta\Delta$Ct method. Significantly higher expression of PK-2 (***$p<0.001$) was evident in nigral tissues following MPTP treatment. Data represent the group mean ± SEM from four mice. Experiments were repeated twice.

**Figure 3.**

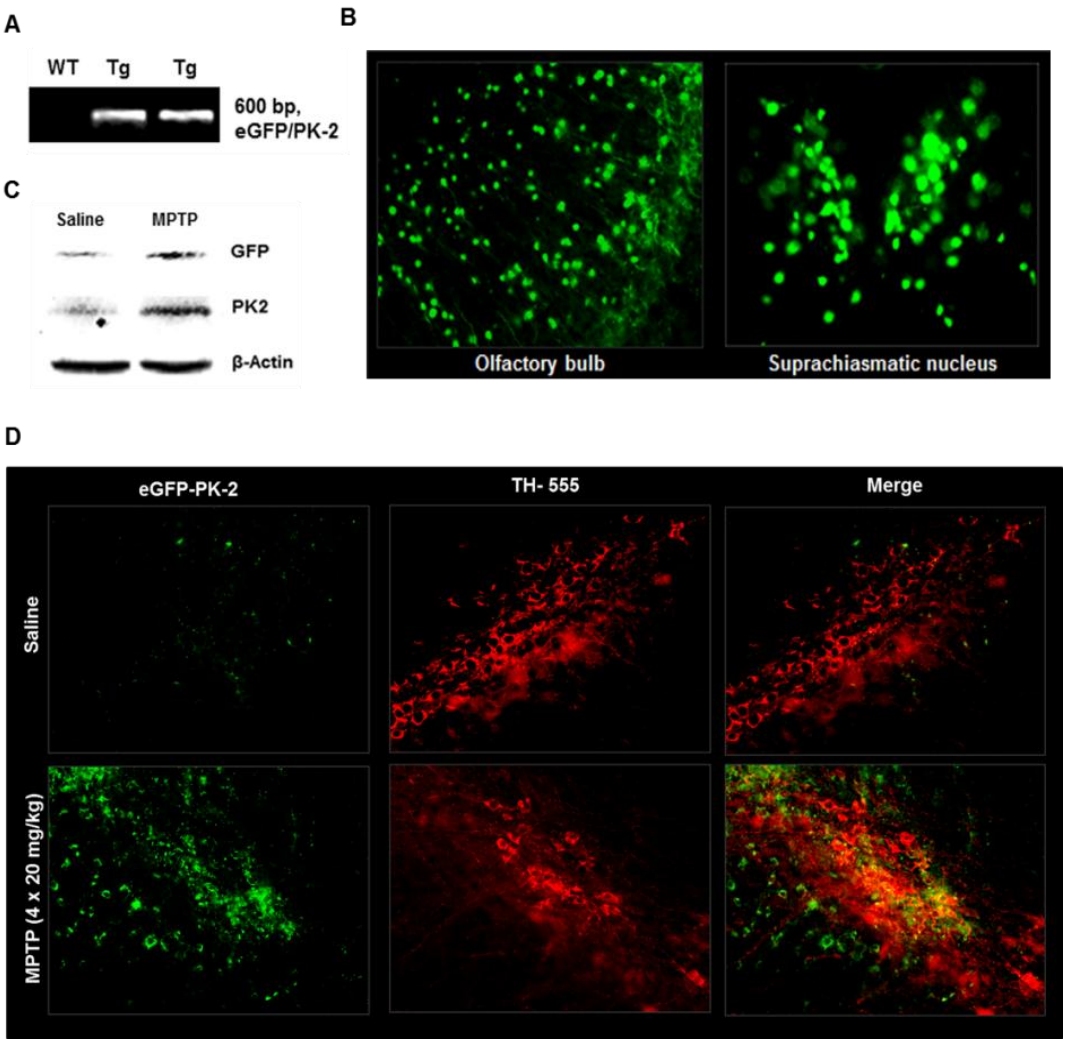



**Figure 3. Visualization of PK-2 induction in vivo using the GENSAT Prok2-EGFP reporter transgenic mouse model.** Prok2-EGFP transgenic mice were generated as part of the GENSAT project on a FVB/N-Swiss Webster hybrid genetic background using bacterial artificial chromosomes (BAC) . **A,** Validation of the PK-2 EGFP reporter transgenic mouse model by genotyping. All mice were genotyped for presence of the transgene prior to experiments according to the recommended PCR protocolusing mouse tail genomic DNA. **B,**

PK-2 expression in the olfactory bulb and suprachiasmatic nucleus (SCN). Expression of the EGFP reporter was determined in the olfactory bulb and the SCN where PK-2 is known be constitutively expressed at high levels. Free-floating sections (30 microns) from the olfactory bulb and the SCN were cut using a freezing microtome, mounted on glass slides and directly visualized for EGFP fluorescence. High levels of EGFP fluorescence was evident in both target regions confirming the veracity of PK-2 expression in the transgenic mice. **C,** Increased EGFP expression in the substantia nigra following MPTP-treatment. Prok2-EGFP transgenic mice were injected with four doses of MPTP (20 mg/kg) at 2 h intervals or saline and were sacrificed 24 h after the last injection. Nigral tissue was dissected and lysates were probed for GFP and PK-2 protein expression by Western blotting. Increased protein levels of GFP along with a concomitant increase in PK-2 protein expression was seen in MPTP-treated mice confirming that PK-2 is highly induced in this mouse strain following MPTP treatment similar to results obtained with C57 black mice. **D,** Visualization of EGFP expression in the Prok2-EGFP transgenic mouse substantia nigra. Free-floating nigral sections (30 microns) were obtained from saline or MPTP-treated transgenic mice sacrificed 24 h after the last injection. Sections were stained for TH on the red channel using an Alexa-555 secondary antibody and EGFP fluorescence was directly imaged on the green channel (488 nm excitation). Increased EGFP expression is evident in the MPTP-treated mouse substantia nigra as seen in the merged image with TH. In saline controls little or no expression of EGFP was evident in these mice similar to results obtained with C57 black mice. Image magnifications are 20X. Representative images are shown and all experiments were repeated at least three times.

**Figure 4.**

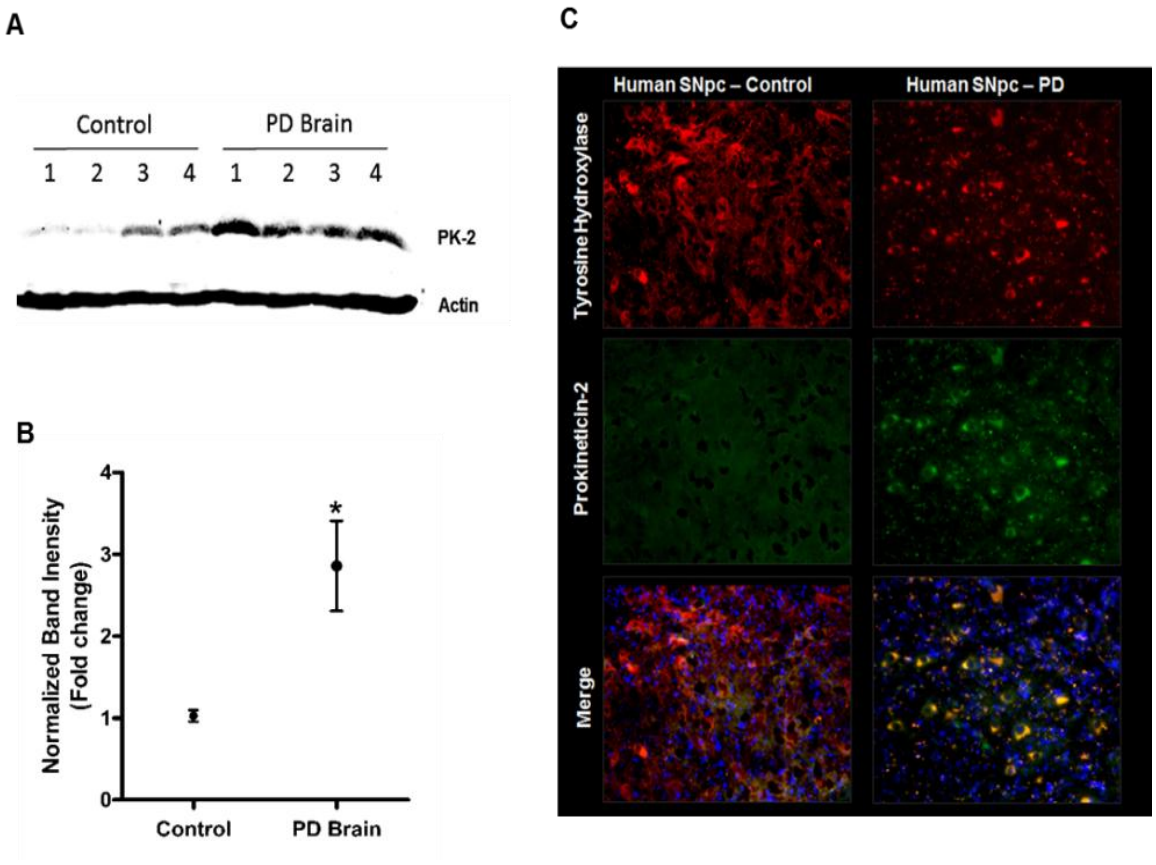



**Figure 4. PK-2 expression is increased in the substantia nigra of human PD patients. A,** Western blot for PK-2 protein levels in PD brain tissue lysates and age-matched controls. Substantia nigra lysates from PD brain tissue and age-matched controls were probed for PK-2 expression using a rabbit polyclonal antibody directed against an internal region of the human PK-2 protein. A total of 50 μg protein was loaded for each sample and actin was used as the internal loading control. Increased levels of PK-2 was evident in most PD samples compared to controls although some variability in PK-2 expression levels was observed in both control and PD patients. A representative blot is shown and experiments were repeated

twice. **B,** Densitometric analysis of PK-2 protein levels. Band intensities for PK-2 were determined from six control and PD samples and were normalized using actin as the internal control. The data was expressed as a fold change over control. Asterisks denote a significant (* $p<0.05$) difference between control and PD samples. **C,** PK-2 immunohistochemistry in human nigral tissue sections. Sections from PD brains and age-matched controls were processed for immunohistochemistry using a rabbit polyclonal antibody that detects human PK-2 (green - Alexa488). Tyrosine hydroxylase (Red - Alexa555) was used as a marker for dopaminergic neurons and Hoechst stain (blue) was used to label the nucleus. PK-2 expression was low or almost absent around melanized dopaminergic neurons in control midbrain nigral sections while high levels of PK-2 was evident in sections from PD brains. More than 90% of the PK-2 positive cells (green) were also TH-positive (red) as seen in the merged image. Representative images are shown and experiments were repeated twice.

**Figure 5.**

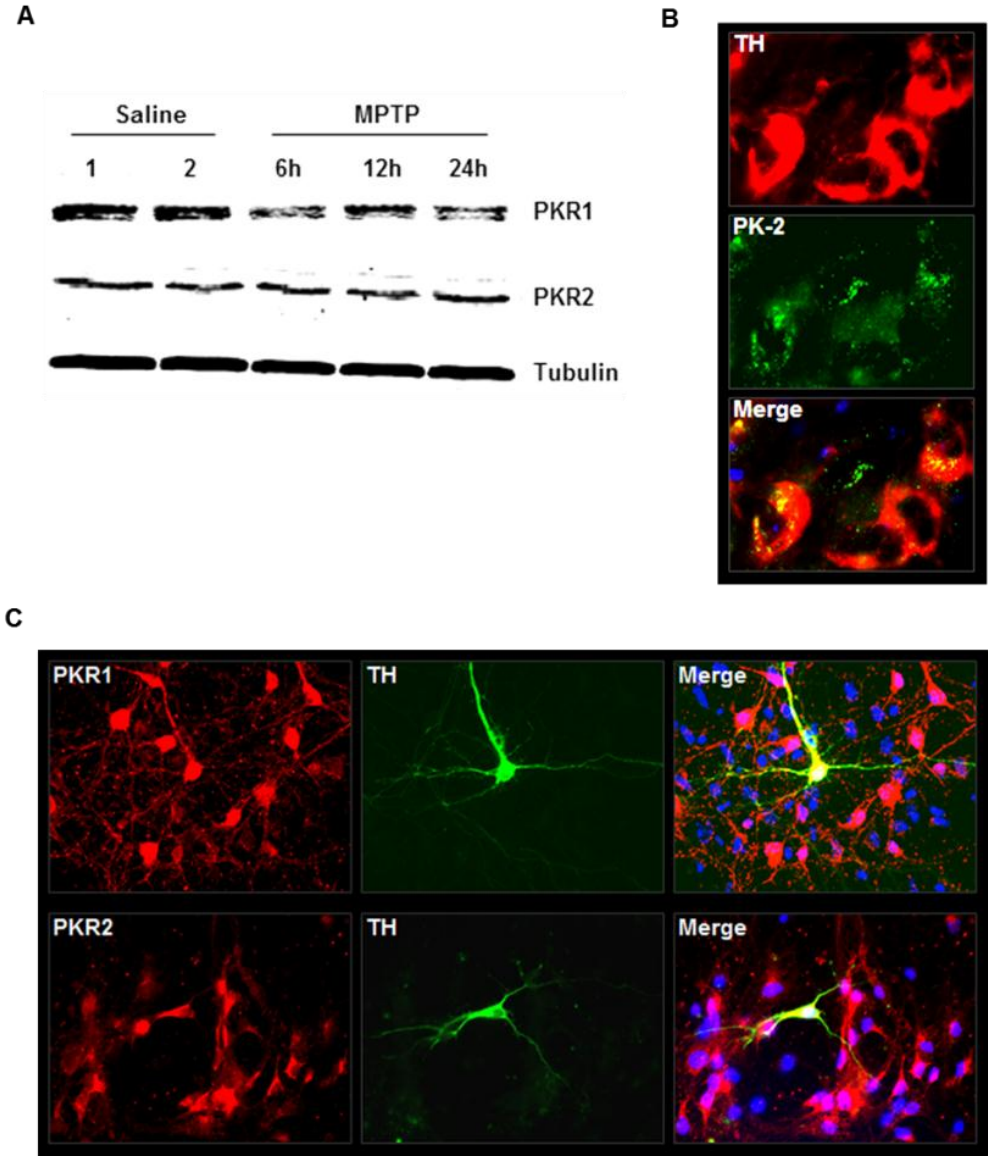



**Figure 5. Expression of prokineticin receptors in the mouse substantia nigra and primary mesencephalic neurons. A,** Western blot for prokineticin receptors PKR1 and PKR2 in mouse nigral tissue lysates. C57 black mice were treated with 4 doses of MPTP (18 mg/kg, i.p.) and sacrificed at the indicated time points. Nigral tissues were dissected and

lysates probed for the two prokineticin receptors PKR1 and PKR2 using rabbit polyclonal antibodies. Tubulin was used as the loading control. Both PKR1 and PKR2 were highly expressed in the substantia nigra. Representative blots are shown and experiments were repeated at least three times. **B,** Expression of PKR2 on substantia nigra dopaminergic neurons. Free floating nigral sections (30 microns) were cut and processed for PKR2 and TH immunohistochemistry. PKR2 (green - Alexa488) was detected using a rabbit polyclonal antibody and TH (red – Alexa555) using a mouse monoclonal antibody. PKR2 expression in nigral sections was evident as punctate staining and was highly expressed on TH-positive dopaminergic neurons as seen in the merged image. Image magnification is 60X. **C,** Expression of prokineticin receptors in primary neuronal cultures. Primary mouse embryonic (E14) ventral mesencephalic neuron cultures were processed for immunocytochemistry at 4 days in vitro. Cells were fixed with 4% PFA for 15 minutes and stained with rabbit polyclonal antibodies to PKR1 and PKR2 (green – Alexa488). Dopaminergic neurons were labeled with TH (red – Alexa555). Species-specific Alexa dye-tagged secondary antibodies were used to detect each primary antibody and Hoechst (blue) was used to stain the nucleus. As seen in the merged image on the right, both PKR1 and PKR2 were highly expressed by TH-positive dopaminergic neurons (merged yellow). PKR1 was also expressed along neuronal processes besides the neuron cell body while PKR2 expression was localized more the neuronal cell bodies. PKR1 and PKR2 were expressed on non-dopaminergic neuronal cells as well as seen from the merged image although several Hoechst-positive neuronal cells were negative for prokineticin receptor expression. Image magnifications are 20X and representative images are shown. Experiments were repeated three times.

**Figure 6.**

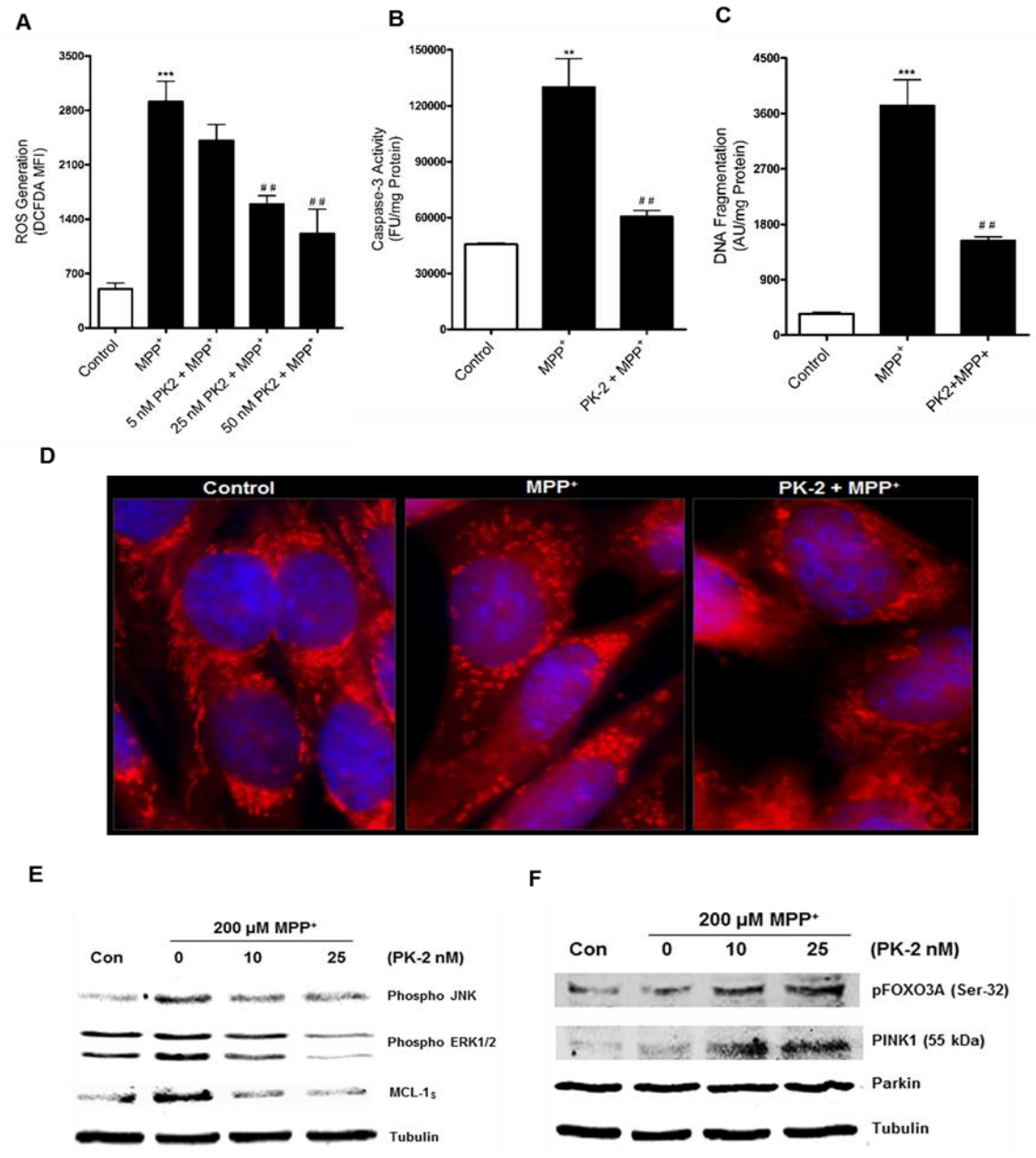



**Figure 6. Exogenous recombinant PK-2 protects N27 cells against MPP+ mitochondrial neurotoxicity and increases PINK-1 protein. A,** Recombinant PK-2 attenuates intracellular ROS generation by induced by $MPP^+$ or co-treated with various doses of recombinant PK-2.

N27 cells were treated with 200 μM MPP+ in 2% serum RPMI for 8 hours and total intracellular ROS levels were determined by quantifying the DCFDA dye fluorescence using a plate reader. MPP+ induced a significant increase in ROS generation which was reduced with PK-2 cotreatment in a dose-dependent manner suggesting that PK-2 signaling via its GPCRs activates survival signaling that protects against $MPP^+$ oxidative stress. **B-C**, PK-2 signaling attenuates $MPP^+$ induced caspase-3 activation and DNA fragmentation. N27 cells were treated with $MPP^+$ (200 μM) or co-treated with PK-2 (25 nM) and cells were collected for caspase-3 activity assays (B) at 8 h and DNA fragmentation analysis (C) at 16 h. Caspase-3 activity was determined using a fluorescent peptide substrate (Ac-DEVD-AFC) and DNA fragmentation was quantified using a sensitive ELISA. The raw data was normalized to the total amount of protein per sample (in mg) determined using a Bradford protein assay and expressed as fluorescence units per mg of protein (FU/mg protein) for caspase-3 activity and absorbance units per mg protein (AU/mg protein) for the DNA fragmentation ELISA. $MPP^+$ treatment induced robust activation of caspase-3 at 8 h (B) and resulted in DNA fragmentation at 16 h (C). Co-treatment with PK-2 significantly attenuated both the activation of caspase-3 and DNA fragmentation induced by $MPP^+$. Data represents the group mean ± SEM. Asterisks denote a significant difference (** $p<0.01$ and *** $p<0.001$) difference between control and $MPP^+$ treatment groups. # # p <0.01 between MPP+ treatment and the PK-2 co-treatment groups. **D,** PK-2 signaling blocks $MPP^+$ induced mitochondrial fragmentation. N27 cells were treated with MPP+ (200 μM) for 16 h in 2% serum media and then incubated with MitoTracker Red dye for 15 minutes to label the mitochondria. Cells were fixed in 4% PFA and the nucleus was stained using Hoechst (blue).

Mitochondrial fragmentation was visualized by fluorescence microscopy using a Nikon TE-2000 fluorescence microscope and images were captured at 60X magnification. $MPP^+$ treatment resulted in highly fragmented mitochondria (center panel) which was attenuated by cotreatement with PK-2 as seen by the presence of an intact mitochondrial network and significantly less fragmented mitochondria. **E,** PK-2 attenuates activation of pro-apoptotic signaling pathways by $MPP^+$. N27 cells were treated with $MPP^+$ (200 μM) or co-treated with PK-2 for 12 h and lysates were probed for the activation of pro-apoptotic signaling pathways. MPP+ treatment induced activation of ERK and JNK as seen by increased phosphorylation at their respective active sites. MPP+ also caused an increase in the levels of the short MCL-1 isoform ($MCL\text{-}1_S$) which is known to drive prop-apoptotic signaling following activation of the mitochondrial cell death pathway. Consistent with the results obtained with caspase-3 activity and DNA fragmentation assays, co-treatment with recombinant PK-2 decreased the levels of active (phospho) JNK and ERK1/2 and also suppressed the increase in $MCL\text{-}1_S$ induced by $MPP^+$.**F,** PK-2 modulates PINK-1 and FOXO-3A signaling to protect against $MPP^+$ neurotoxicity. Lysates from N27 cells treated with either $MPP^+$ or co-treated with PK-2 were probed PINK-1, PARKIN and phospho FOXO-3A (Thr-32) by Western blotting. Cells co-treated with PK-2 expressed higher levels of the PINK-1 protein and increased phospho FOXO-3A (Thr-32) compared to either untreated controls or $MPP^+$ treated cells suggesting that these survival signaling pathways might be relevant in PK-2 mediated protection of N27 cells from MPP+ induced cell death. Representative blots are shown and experiments were repeated twice.

**Figure 7.**

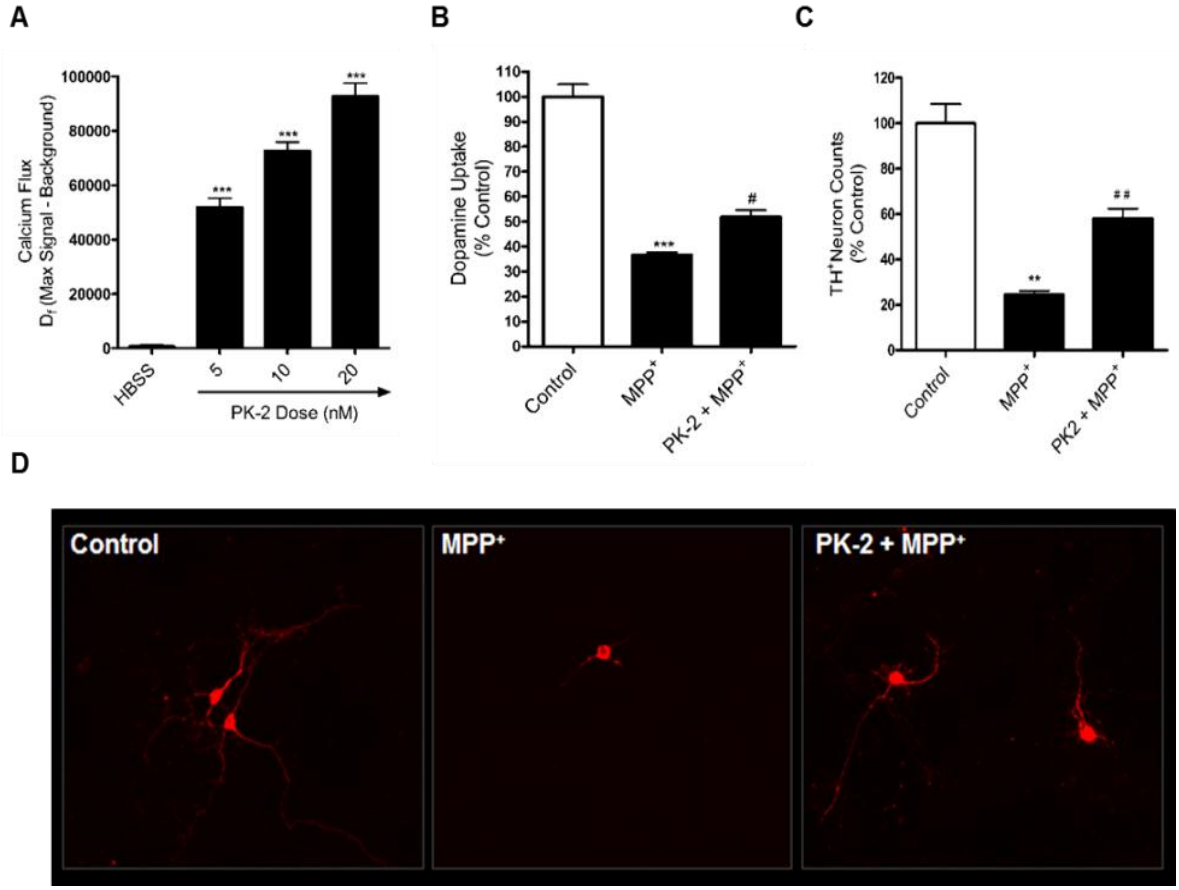



**Figure 7. PK-2 protects primary dopaminergic neurons from $MPP^+$ neurotoxicity. A, Calcium mobilization by PK-2 in primary EVM neuronal cultures**. Primary EVM neuron cultures were treated with nanomolar doses of recombinant PK-2 and the calcium flux quantified using the Fluo-4 NW calcium assay kit. Control cells were treated with calcium-free HBSS. Calcium-flux data was expressed as the net change in fluorescence signal ($D_f$) representing the maximum signal after baseline subtraction for each dose of PK-2. As shown, PK-2 mobilized calcium in a dose-dependent manner in primary neuron cultures, which also express both prokineticin receptors (Fig. 4). Data represent the group mean ± SEM. Asterisks

denote a significant (*** $p<0.001$) difference between HBSS-treated controls and PK-2 treated groups. **B,C** Dopamine uptake assay and TH+ neuron counts in primary EVM neuronal cultures. Primary EVM cultures were grown in neurobasal medium in 24 well plates on poly-D-lysine coated coverslips and treated 4 days after plating (DIV-4). Cells were treated with $MPP^+$ (5 μM) for 24 h or co-treated with recombinant PK-2 (25 nM). PK-2 was added at the beginning of the treatment and again 12 hours later. For dopamine uptake assays (B) cells were washed in Krebs-Ringer buffer and then incubated with 5 μM [$^3$H]-Dopamine for 20 minutes at 37°C. Cultures were then washed in ice-cold KRB, dissolved in 1 N NaOH and mixed with scintillation fluid. Radioactivity counts were determined using a Tricarb scintillation counter. The non-specific uptake of dopamine determined in paralell wells in the presence of mazindol was subtracted and the specific dopamine uptake was plotted for each group as percent control (B). For TH-positive neuron counts, cultures were fixed in 4% PFA at the end treatment and dopaminergic neurons were visualized by TH-immunocytochemistry using an Alexa-555 secondary antibody (red). Cells from eight independent fields were counted from each group in triplicate and data was expressed as percent control (C).Data represent the group mean ± the SEM. Asterisks denote a significant (*** $p<0.001$) difference between the control and $MPP^+$ treated group. # # denotes $p<0.01$ and # denotes $p<0.05$ PK-2 between the $MPP^+$ and PK-2 co-treatment groups. Co-treatment with PK-2 significantly improved dopaminergic neuron survival and function compared to $MPP^+$ treatment alone. **D,** Representative images of dopaminergic neurons in primary EVM cultures. Images were acquired at 20X magnification. As shown, MPP+ treatment caused extensive degeneration of dopaminergic neurons which was attenuated in the presence of recombinant PK-2

# CHAPTER VI

# GENERAL CONCLUSION

This section presents a general overview of the results and findings described in the thesis herein, with a special emphasis on future directions and overall implications of these findings for the pathogenesis and progression of Parkinson's disease. The major findings pertaining to each research manuscript and their specific implications are covered in the 'results and discussion' sections of the relevant chapter.

**Distinct PKCδ activation mechanisms in dopaminergic neurons and microglia**

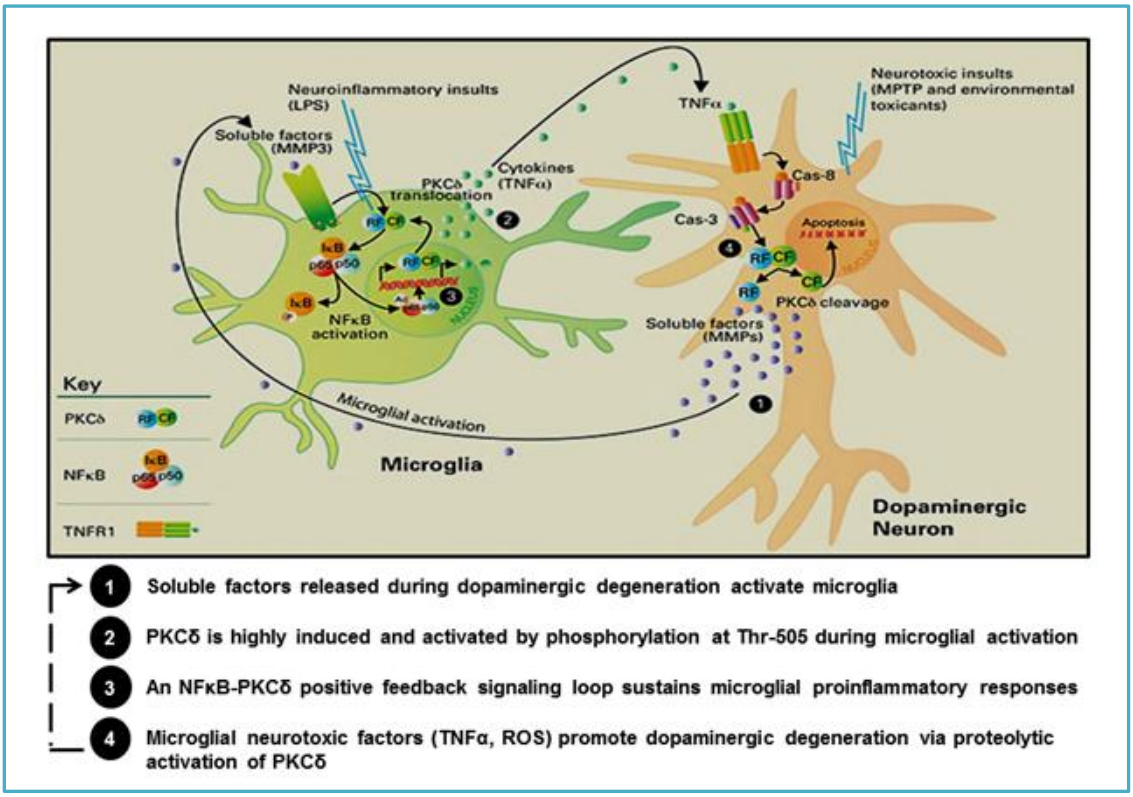
Taken together, the results from chapter 2 and 3 demonstrate that PKCδ is activated in both dopaminergic neurons and microglial cells by two distinct mechanisms. In dopaminergic neurons, PKCδ is proteolytically activated by caspase-3 via the extrinsic apoptotic cascade downstream of TNFR1 signaling and promotes apoptosis. In microglial cells however PKCδ protein expression is highly induced following microglial activation by LPS, TNFα or aggregated alpha-synuclein with a concomitant increase in kinase activity, indicative of a classical PKC activation mechanism involving phosphorylation at the activation loop. While proteolytic activation of PKCδ in dopaminergic neurons promotes apoptosis, the induction of PKCδ in microglial cells promotes activation and proinflammatory responses. Further, the induction of PKCδ in microglia appears to be a common downstream target of distinct microglial activation stimuli, LPS via TLR4, TNFα through its TNF receptors and aggregated alpha synuclein via phagocytosis (Zhang et al., 2005b).

Previous studies from our laboratory have shown that during dopaminergic cell death induced by oxidative stress or neurotoxic insults such as MPP+, proteolytic activation of PKCδ occurs via the intrinsic mitochondrial apoptotic pathway and promotes apoptosis. Proteolytic activation of PKCδ by TNFR1 signaling lends further support for a direct neurotoxic role for TNFα signaling on dopaminergic neurons in addition to potentiating microglial neurotoxicity. Our finding that PKCδ is proteolytically activated in the substantia nigra following stereotaxic LPS infusion demonstrates that this signaling pathway is relevant to dopaminergic degeneration initiated by inflammatory insults in vivo. It also indicates that proteolytic activation of PKCδ could be a common downstream mediator during dopaminergic cell death induced by diverse neurotoxic signaling mechanisms that converge on the extrinsic and mitochondrial apoptotic pathways.

A TNFα-PKCδ signaling loop between microglia and neuronal cells during progressive dopaminergic degeneration and sustained microglial activation is also evident from these findings (Figure 1). Dopaminergic degeneration is initiated by various neurotoxic factors and injured dopaminergic cells release factors such as MMP-3 and alpha synuclein aggregates, which can activate microglia and induce the upregulation of PKCδ. PKCδ most likely regulates the proinflammatory response in the microglia by activating the p65/NFκB pathway. Activation of the NFκB pathway leads to the sustained upregulation of PKCδ in these cells and the induction of proinflammatory mediators including iNOS and TNFα (Jin et al., 2011). The microglial TNFα can then act on compromised dopaminergic neurons by signaling through its TNFR1 receptor and lead to activation of caspase-3 via caspase-8. Activation of caspase-3 leads to downstream activation of PKCδ in dopaminergic cells

resulting in proteolytic cleavage of PKCδ into its catalytic fragment (CF) and regulatory fragment (RF). The catalytic fragment translocates to the nucleus and promotes cell death in dopaminergic neurons. This leads to release of further soluble mediators from dopaminergic neurons which activate microglial cells, potentially amplifying the signaling loop and leading to sustained reactive microgliosis (Block et al., 2007a)

**Figure 1: Distinct mechanisms of PKCδ activation in microglia and dopaminergic cells**




**PKCδ substrates and signaling pathways**

The downstream substrates of PKCδ proapoptotic signaling have been identified including laminin, DNA-PK and histone H1 in the nucleus (Kanthasamy et al., 2003b) and

these are likely to be involved in PKCδ signaling during TNF-induced dopaminergic cell death. The upstream signaling pathway that results in proteolytic activation of PKCδ by TNF in N27 cells remains to be defined. Since there is substantial cross talk between the extrinsic and intrinsic apoptotic pathways downstream of TNF death receptor signaling via t-bid (Chen and Goeddel, 2002), TNF signaling could synergize with other neurotoxic mediators that initiate dopaminergic degeneration via mitochondrial apoptosis, with PKCδ being a common downstream target (Korsmeyer et al., 2000; Anantharam et al., 2002; Kaul et al., 2003b; Afeseh Ngwa et al., 2009). This could be one potential mechanism by which TNF can sustain reactive microgliosis and promote dopaminergic cell death resulting in progressive neurotoxicity (Block et al., 2007a).

The precise signaling mechanism and downstream targets of PKCδ during microglial activation are currently under investigation. However, potential downstream targets for regulation by PKCδ can be inferred based on the known substrates and functions of PKCδ in innate immune cells. Both NFκB/p65 and the p47-PHOX subunit of the NADPH oxidase complex are substrates for PKCδ in other cell types (Cheng et al., 2007; Mut et al., 2010) and regulate multiple arms of the microglial activation response including cytokine release, superoxide production and iNOS activation. Our findings suggest that microglia isolated from PKCδ knockout (PKCδ-/-) mice have attenuated nitric oxide and intracellular ROS generation, as well as reduced levels of proinflammatory cytokines and chemokines compared to microglia from wildtype (PKCδ +/+) mice. This suggests that NFκB or NADPH oxidase activity may be affected in microglia isolated from PKCδ knockout mice. The serine/threonine and tyrosine phosphorylation status of PKCδ during microglial activation is

also currently being investigated and may provide novel insight into understanding the detailed mechanism of PKCδ activation in these cells and help define substrate specificity of the kinase.

**PKCδ signaling in regulation of systemic inflammatory responses**

Recent studies have shown that the brain has a heightened sensitivity to systemic inflammatory insults. In particular, the systemic injection of LPS causes an elevation of TNF alpha in the brain for up to ten months, while the peripheral levels of TNF return to basal levels in less than a week. Further, TNFα signaling was found to be important for the transfer of peripheral systemic inflammation into the brain (Qin et al., 2007). We obtained similar results with LPS-injected PKCδ wildtype mice, which showed elevated levels of TNFα mRNA in the brain (20- to 40-fold over saline controls). Further, these mice showed significant LPS-induced sickness behavior typical of LPS activation at 48 hours. PKCδ knockout (PKCδ -/-) mice however, recover rapidly from LPS-induced sickness and are almost normal at 48 hours with behavioral parameters similar to those of saline controls. Importantly, the brain TNF mRNA levels are significantly reduced in these mice (two to threefold over saline controls) suggesting an important role for PKCδ signaling in mediating the systemic inflammatory response to LPS and the heightened inflammatory state in the brain. Our results from the behavioral studies with PKCδ knockout mice using the systemic LPS injection model are intriguing in the light of similar results published recently in a model of lung injury induced by polymicrobial sepsis (Kilpatrick et al., 2011). Targeted inhibition of PKCδ in this model effectively prevented lung injury following polymicrobial sepsis suggesting that PKCδ is a crucial regulator of proinflammatory events in the lung. The

specific immune cell types and the mechanisms involved are yet to be defined although based on the existing literature, neutrophils and monocytes/macrophages are likely to be the primary innate immune cells involved

**Dual neuroprotective outcome of targeted PKCδ inhibition**

The simultaneous activation of PKCδ in both microglia and dopaminergic neurons by distinct mechanisms during dopaminergic degeneration suggests that the use of small-molecule inhibitors could be an effective therapeutic strategy against dopaminergic degeneration. Targeted kinase inhibition of PKCδ using TAT-PKCδ peptides or small molecule inhibitors has shown to be effective in inhibiting PKCδ signaling in vivo (Zhang et al., 2007c; Kilpatrick et al., 2011). Importantly, our findings reported here suggest that inhibition of PKCδ could result in significant dopaminergic neuroprotection by acting on both the microglial neurotoxic response and directly preventing dopaminergic neuron loss. Targeted PKCδ inhibition could therefore be very effective at shutting down the self-perpetuating neurotoxicity that is believed to drive sustained reactive microgliosis and dopaminergic neurotoxicity. Our neuroprotection experiments with the MPTP model in PKCδ knockout mice demonstrate this mechanistically. In PKCδ knockout mice, the microglial activation response in the substantia nigra is blunted in both LPS and MPTP models. The nigrostriatal system is also effectively preserved in these mice in the MPTP model of PD with significant protection in terms of neurochemical and behavioral parameters. Similar results were obtained in previous studies from our research group using the PKCδ inhibitor rottlerin, demonstrating the feasibility of this approach. Although the

neuroinflammatory responses were not evaluated in that study, significant protection of the nigrostriatal system against MPTP was evident in rottlerin-treated mice.

Dominant negative inhibitors to soluble TNFα have recently shown tremendous efficacy in protecting dopaminergic neurons from nigrostriatal degeneration in animal models of PD. This is likely due to a similar dual-neuroprotective effect of TNF inhibition that prevents both direct dopaminergic neurotoxicity and a potentiation of the microglial inflammatory responses. Our results here identify that PKCδ signaling could act downstream of TNF in dopaminergic neurons and upstream of microglial TNFα release. Taken together, the findings reported here provide further rational for targeting PKCδ in PD and could be an effective therapeutic strategy to mitigate microglial neurotoxicity and progressive dopaminergic degeneration.

**Relevance of PK2 signaling to Parkinson's disease**

The findings reported in chapter V demonstrate that PK2 is highly induced following dopaminergic neuron injury in cell culture and animal models of PD and is also elevated in the postmortem human PD brains. These findings suggest intriguing new functions for PK2 in the pathophysiology of Parkinson's disease and neurodegeneration in general by providing the first evidence for the induction of PK2 expression in neurons following injury or in a neurodegenerative disease. We believe that the induction of PK2 in the MPTP model of PD is most likely mediated by oxidative stress-induced activation of the basic helix-loop-helix transcription factor HIF1α that has previously been shown to occur in the MPTP model of PD (Lee et al., 2009). HIF1α can activate E-box elements, which are abundantly expressed on the PK2 promoter proximal to the transcription start site. This hypothesis is being tested

using HIF1 activators and inhibitors in cell culture and animal models of PD. Our preliminary results using HIF1α agonists in N27 cells suggest that PK2 can be induced in dopaminergic cells by HIF1α activation. Further studies are being performed to validate this hypothesis in vitro and in the MPTP mouse model.

**PK2 as a potential neuroprotective factor for dopaminergic neurons**

We verified that receptors for PK2 are expressed on primary dopaminergic neurons, N27 dopaminergic cells and in the mouse substantia nigra confirming that the dopaminergic neurons themselves could be the target of secreted PK2 in the nigra. Our functional studies demonstrate that PK2 protects dopaminergic neurons in vitro from $MPP^+$ induced oxidative stress and cell death by a novel mechanism involving the upregulation of the mitochondrial survival kinase PINK1. The activation of two important survival kinases, the Akt and MAPK pathways, have been consistently reported in several studies using different cell types from neurons to hematopoietic cells. In almost all studies to date, prokineticin signaling has been associated with cell survival and differentiation or cell migration and activation. To date, there is no evidence linking prokineticin signaling with apoptotic or other cell degenerative signaling pathways. Rather, PK2 signaling in the CNS has been associated with adult neurogenesis in the subventicular zone and olfactory bulb suggesting that PK2 can function as a neurotrophic factor and a chemoattractant in the brain. Interestingly, receptors for prokineticins, (particularly PKR2) are constitutively expressed across the brain at high density even in areas such as the midbrain where no prokineticin ligand is normally detectable. It is possible that PK2 is induced locally at sites of neuronal injury or degeneration in the brain and functions as a neuroprotective factor by signaling through

PKR2. Taken together, the results of our mechanistic studies clearly demonstrate that PK2 can effectively protect against mitochondrial oxidative stress and neurotoxicity in dopaminergic neurons. These findings thus have immense therapeutic implications for targeting the prokineticin GPCRs expressed on these cells using PK2 agonists

**Potential functions of PK2 signaling in microglia and astrocytes**

Both microglia and astrocytes have been shown to express prokineticin receptors (Koyama et al., 2006). Therefore, PK2 released from dopaminergic neurons may also play a role in regulating microglia and astrocyte responses. Based on previous studies and known functions of PK2 on immune cells, it is likely that the PK2 released from injured dopaminergic neurons is a chemoattractant or aproliferation signal for microglia and astrocytes in the substantia nigra. Secreted PK2 can therefore direct the migration of astrocytes and microglia to sites of neuronal injury and facilitate neuron survival by potentially eliciting glial neurotrophic factors. PK2 can also function as a pro or anti-inflammatory mediator on microglia and astrocytes and thus regulate dopaminergic neuron-glia interactions. Our preliminary studies suggest that indeed PK2 functions as a chemoattractant for microglia and astrocytes in vitro. Current studies are focused on establishing the overall neuroprotective functions of PK2 signaling in vivo using adenoviral overexpression of PK2 in the mouse substantia nigra and the MPTP model of PD. The effect PK2 on astrocyte and microglial migration and a potential mechanisms of neuroprotective glial responses is also being evaluated in cell culture and animal models.

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